Xenoestrogen levels in milk – bioavailability and relevance as breast cancer risk factors.

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Abstract

Hormones are key control molecules fundamental to most biochemical functions in the body. The endocrine system is responsible for the synthesis, regulation and secretion of sex hormones into the circulatory system to control target organs, tissue and cells through receptor mediated responses. The balance between two key steroid hormones, 17β-estradiol (E2) and testosterone, the female and male sex hormones respectively is critical for fetal growth and development. A disruption to this tightly regulated system may lead to implications such as early onset of puberty in girls, decreased sperm count, and increased risk of breast cancer in postmenopausal women. Xenoestrogens (from the Greek xenos meaning stranger) are foreign compounds that have structural similarities to E2 and although the binding site for E2 is very specific, they have the ability to bind to estrogen receptors (ERs) but not as well, resulting in less of a biological response. Each xenoestrogen has a different affinity for ERs resulting in various cellular responses – also known as estrogenicity.

There has been an increasing interest in the concentration of xenoestrogens in cow's milk due to the possible effects consuming these compounds may have on the population. Here, this study utilised and evaluated a method for extracting endogenous estrogens (E2) and xenoestrogens (17α-ethinyl estradiol; EE2, formononetin, genistein, daidzein, equol, bisphenol A; BPA and zearalenone) from cow's milk. The performance of this method was acceptable with recoveries ranging from 62% (BPA) to 95% (EE2), limits of determination ranging from 0.0025 mg/L (daidzein) to 0.1 mg/L (E2) and limits of quantification of similar values ranging from 0.05 mg/l (zearalenone) to 1 mg/L (E2).

During the duration of the current study articles were published citing tests that found trace amounts of the herbicide, glyphosate, in Ben and Jerry's ice cream in the United Kingdom. These findings and published literature on glyphosates ability to induce human breast cancer cell growth lead to question the presence of glyphosate in New Zealand cow's milk. A previously accepted extraction method was used to determine the presence of glyphosate, due to the low concentrations published, direct inject probe

mass spectroscopy was utilised. In sampled New Zealand cow's milk from the Canterbury region (n = 5), no mass ion consistent with glyphosate was identified.

This extraction method was used to assess the safety of New Zealand cow's milk in an estrogenic mimic context. In sampled New Zealand cow's milk from the Canterbury region (n = 30), BPA was identified in 9 samples at concentrations ranging from 0.012 mg/L to 0.066 mg/L, daidzein was identified in 1 sample at a concentration of 0.026 mg/L and genistein was identified in 1 sample after treatment with sulfatase/β-glucuronidase at a concentration of 0.062 mg/L. Based on the values from this study a person who drinks the New Zealand population's average amount of milk (133 mL) would not be exposed to concentrations of each compound that exceeds their acceptable daily intakes (BPA, 0.05 mg/kg bw; genistein, 1.5×10^{-4} mg/kg bw; and daidzein no data (based on structural similarities to genistein it was assumed daidzein would have a similar acceptable daily intake value)). In New Zealand, cow's milk is an essential part of the diet providing key minerals and nutrients, the findings in the present study suggest that New Zealand cow's milk is safe. However, future studies could investigate whether more sensitive analytical techniques could detect other possible xenoestrogens at lower concentrations compared to high performance liquid chromatography – diode array detector (HPLC-DAD).

Abbreviations

3β-HSD	3β-Hydroxysteroid dehydrogenase
ADI	Acceptable daily intake
AF	Activation functions
AMPA	Aminomethylphosphonic acid
ARfD	Acute reference dose
BPA	Bisphenol A
BST	Bovine somatotropin
CRI	Crown Research Institutes
DAD	Diode array detector
DBD	DNA binding domain
DCM	Dichloromethane
DDE	Dichlorodiphenyldichloroethylene
DDT	Dichlorodiphenyltrichloroethane
DES	Diethylstilbestrol
E1	Estrone
E1S	Estrone sulphate
E2	17β-Estradiol
E3	Estriol
EE2	17α-Ethinylestradiol
EPS3P	5-Enolpyruvylshikimate-3-phosphate
EPSPS	5-Enolpyruvylshikimate-3-phosphate synthase
EREs	Estrogen response elements
ERs	Estrogen Receptors
ERα	Estrogen Receptor-α
ERβ	Estrogen Receptor-β
FDA	Food and Drug Administration
FSH	Follicle-stimulating hormone
HDPE	High-density polyethylene
HPLC	High performance liquid chromatography
HPLC-DAD	High performance liquid chromatography – diode array detector
HPLC-MS	High performance liquid chromatography – mass spectrometry
HRT	Hormone replacement therapy

LBC	Ligand binding cavity
LBD	Ligand binding domain
LC-MS	Liquid chromatography – mass spectrometry
LH	Luteinizing hormone
LLE	Liquid liquid extraction
LOD	Limit of determination
LOQ	Limit of quantification
MRL	Maximum residue limit
NCCP	National Chemical Contaminants Programme
NMR	Nuclear mass resonance
NOAEL	No observable adverse effect level
NP	Nonylphenol
ODS	Octadecylsilane
P450scc	Cytochrome P450 side-chain cleavage enzyme
PCBs	Polychlorinated biphenyls
PDB	Protein Data Bank
PEP	Phosphoenolpyruvate
PP	Polypropylene
QuEChERS	Quick, easy, cheap, effective, rugged, safe
RCS	Red clover silage
REP	Relative estrogenic potency
RMSD	Root-mean-square deviation
S3P	Shikimate-3-phosphate
SHBG	Sex-hormone binding globulin
SPE	Solid phase extraction
TDI	Tolerable daily intake
t_R	Retention time
USA	United States of America
UV	Ultraviolet
WCS	White clover silage
WHO	World Health Organization
WWTP	Wastewater treatment plant
XP	Extra precision
λ	Wavelength

Chapter 1 – Introduction

Chapter 1 – Introduction

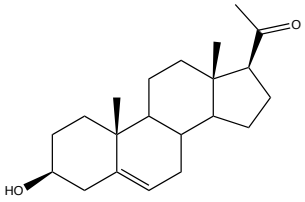
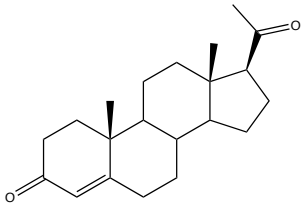
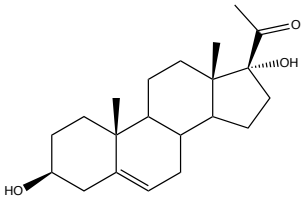
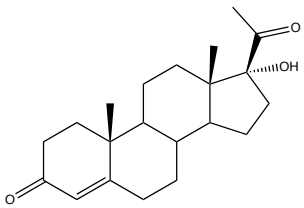
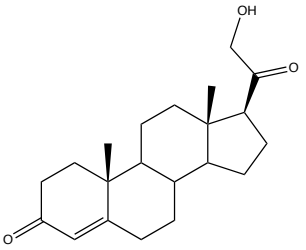
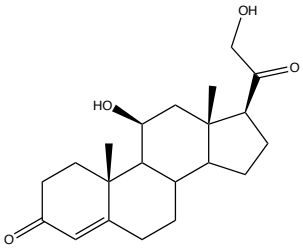
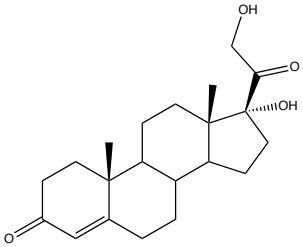
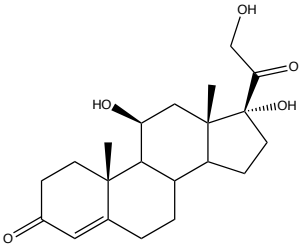
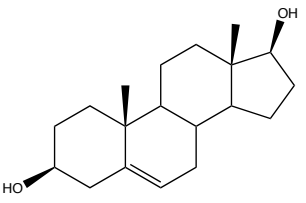
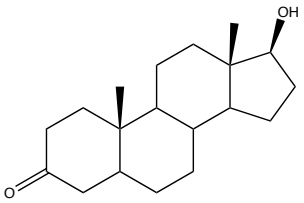
1.1 Brief Overview

The broad ranging biological importance of steroid hormones is well understood; for example, steroid hormones have key roles in metabolism, growth and development, and reproduction.[1-6] Additionally, compounds have been shown to mimic one of the natural estrogens, 17β-estradiol (E2; Fig. 1.1), and bind to the estrogen receptors (ERs) estrogen receptor α (ERα) and estrogen receptor β (ERβ). [7, 8] Binding to the ERs can elicit a range of responses which can, in turn, lead to adverse effects such as an increased risk of some cancers (e.g. breast and ovarian), and reproductive and developmental abnormalities.[5, 9] It is possible that a large exposure to these estrogen mimicking compounds are via the human diet, particularly the dietary staples (e.g. milk, bread in New Zealand).[5, 10, 11] Dietary staples are an important part of the human diet because they provide a major source of energy and contain nutrients and minerals that the body requires. Cow's milk is a dietary staple and is consumed globally, likely due to the health benefits it exhibits (e.g. increased bone strength from calcium consumption via milk). However, previously reported studies in several countries (e.g. USA, China) show the presence of estrogen mimics in milk. [12-22] Despite this finding, only a limited range of studies have been conducted that explore the presence of endogenous estrogens and xenoestrogens in New Zealand cow's milk. This is surprising because New Zealand is one the largest milk exporter in the world.

1.2 Steroid Hormones and the Endocrine System

Steroid hormones are important in control mechanisms for many biological functions, including, but not limited to, metabolism, inflammation, growth and development, immune function, and reproduction.[1] The biosynthesis and release of steroid hormones is the responsibility of the endocrine system; once biosynthesised, they are secreted into the circulatory system and extracellular fluids to be carried to organs, tissues and cells.[1, 2] Once they reach their destination they modulate cellular activity through receptor-mediated responses.[1, 2] There are two classes of steroid hormones; sex steroids and corticosteroids (Fig. 1.1). Corticosteroids are produced in the adrenal cortex of

vertebrates; for example, the biosynthesises of cortisol in response to stress. Whereas, sex steroids are produced by the gonads (ovaries or testes), adrenal glands, or through conversion of other sex steroids in other tissues (e.g. adipose tissue, liver).[3, 4] Two examples of sex steroids are the major female hormone E2, and the major male hormone testosterone. The levels of steroid hormones are tightly regulated in the body by feedback mechanisms; in particular their production and degradation. These are highly controlled because of their narrow activity characteristics; for example, if E2 is present in excess, it will lead to the down regulation of enzymes responsible for the synthesis of E2.[23] An important time that these hormones are tightly controlled is during the formation of the fetus' sex characteristics (e.g. reproductive organs such as the testis and ovaries).[5, 23] Development of these organs is reliant on the balance between the male and female hormones (testosterone and E2 respectively).[5] If the balance of these hormones are upset, growth and development changes might result (e.g. adenocarcinoma, cryptorchidism).[5] The actions of these hormones are receptor mediated; therefore these effects (e.g. adenocarcinoma, cryptorchidism) can occur by an increase of binding to a hormone receptor. These receptors can be either extracellular or intracellular.[6] Extracellular or cell surface receptors are signal transducers; an extracellular signal, i.e. the hormone, binds and activates a receptor leading to a cellular response.[6] Therefore, the feedback mechanism to control the synthesis of hormone is critical for normal growth and development.

Progestagens	 Pregnenolone  Progesterone  17α-Hydroxypregnenolone  17α-Hydroxyprogesterone
Corticosteroids	 11-Deoxycorticosterone  Corticosterone  11-Deoxycortisol  Cortisol
Androgens	 Androstenediol  5α-Dihydrotestosterone

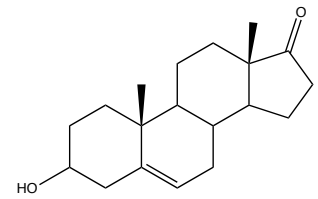
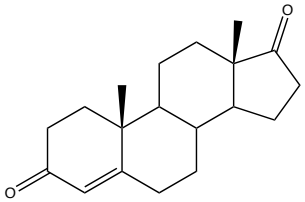
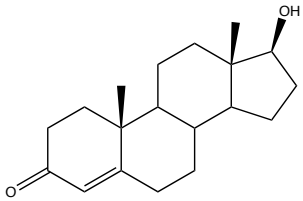
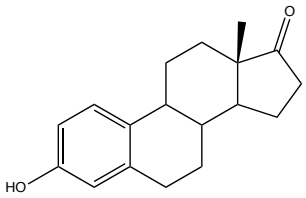
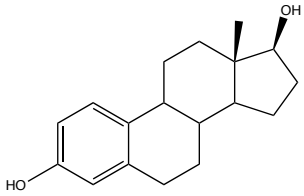
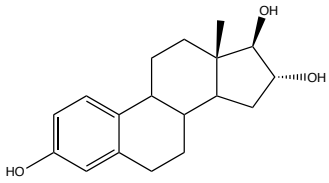
Androgens cont.	 Dehydroepiandrostenedione  Androstenedione  Testosterone
Estrogens	 Estrone (E1)  17β-Estradiol (E2)  Estriol (E3)

Figure 1.1: The key sex steroids and corticosteroids.

1.2.1 Biosynthesis of Steroid Hormones

There are three specialised regions that synthesise steroid hormones in the endocrine system, namely the adrenal cortex, ovary, and testis. Release of hormones from these organs are controlled by specific extracellular hormones and activators initiating the intracellular processes, thus changing the steroid output.[24] For example, the hypothalamus biosynthesises hormones which stimulate the anterior pituitary gland.[25] In turn, the anterior pituitary gland releases the thyroid stimulating hormone, which then stimulates the thyroid gland to produce the hormones T_3 and T_4.[25] As the blood

concentration of T_3 and T_4 rise, they inhibit both the pituitary and hypothalamus in a negative feedback loop so controlling the thyroxine hormone levels and thus activity.[25] While T3 and T4 are not steroid hormones they are a useful example of the mechanism and importance of tight control. The steroid hormones are similarly regulated by a complex array of feedback mechanisms.

The biosynthesis of the steroid hormones begins with the conversion of cholesterol to pregnenolone by cytochrome P450 side-chain cleavage enzyme (P450scc) located in the inner mitochondrial membrane of steroidogenic cells (Fig. 1.2).[26, 27] Pregnenolone exits the mitochondrion and is transported to the endoplasmic reticulum where it is converted to progesterone by 3β-hydroxysteroid dehydrogenase (3β-HSD; Fig 1.2) – an important feedback controlled biosynthetic enzyme.[26, 27] Some steroidogenic cells possess a mitochondrial form of 3β-HSD which converts pregnenolone to progesterone before it leaves the mitochondrion.[27] However, in most species and steroidogenic tissues, the conversion of pregnenolone to progesterone is carried out in the microsomal compartment.[27] In the microsomal compartment, progesterone is converted to an array of other steroids with the assistance of a contingent of enzymes in those tissues; for example, ovarian theca cells secrete androgens which are converted to E2 by ovarian granulosa cells.[27] Likewise, testicular Leydig cells synthesise the androgen, testosterone.[27] The final step in the biosynthesis of estrogens is the conversion of the androgens, namely testosterone and androstenedion, to E2 and E1 respectively (Fig. 1.2).[26] The enzyme responsible for this is aromatase (CYP19A1), a member of the cytochrome P450 superfamily.[28] The distribution of this cytochrome P450 member is wide; located in the gonads, brain, placenta, and adipose tissue.[28] The wide distribution of aromatase means that it might play an important role in the balance of the androgens and estrogens.

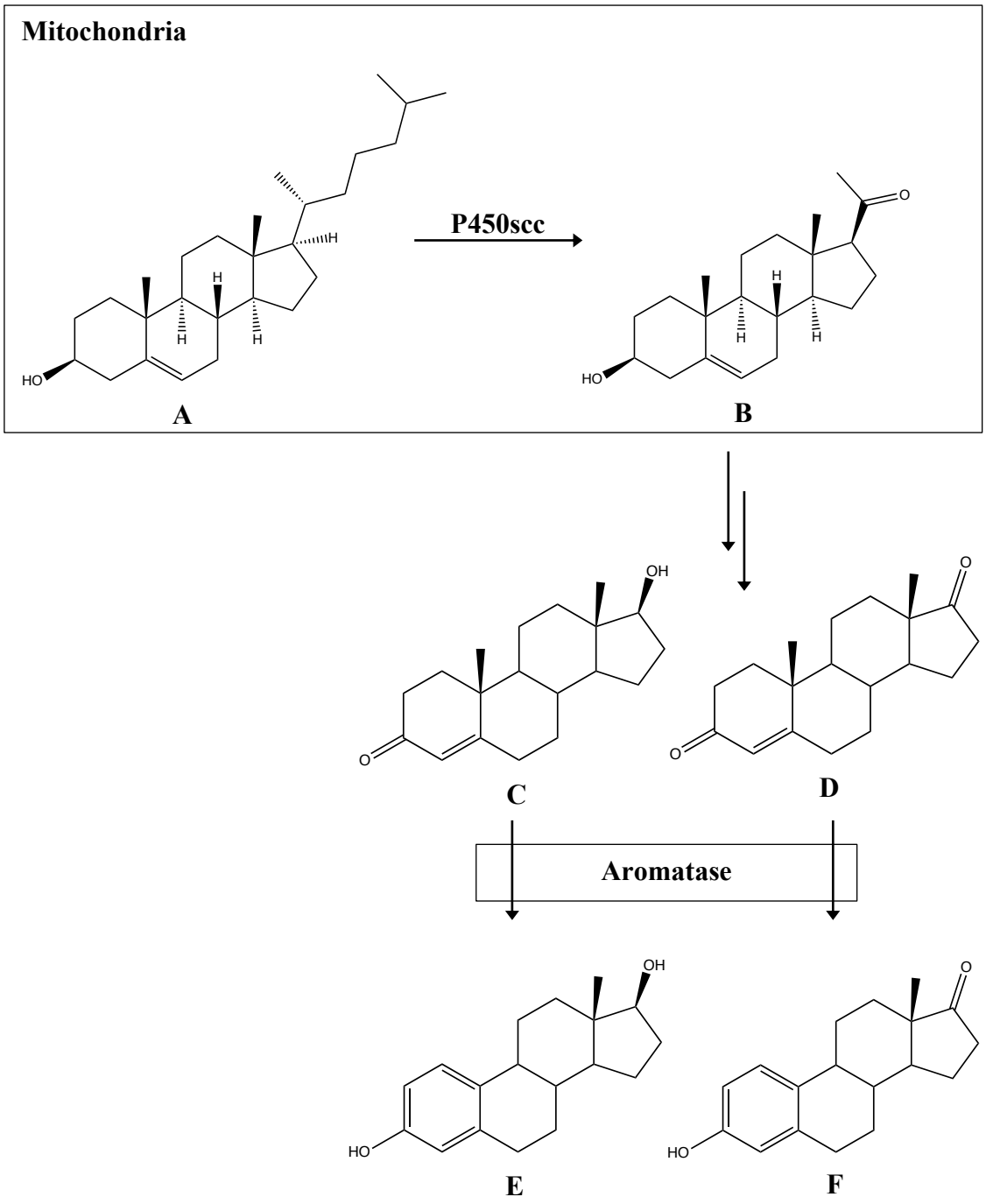


Figure 1.2: The first step in steroid hormone biosynthesis, converting cholesterol (**A**) to pregnenolone (**B**) catalysed by P450scc in the mitochondrion. Pregnenolone transported out of the mitochondria where it is converted to the androgens testosterone (**C**) and androstenedione (**D**) by a contingent of enzymes. These are finally converted to the estrogens E2 (**E**) and E1 (**F**) by the cytochrome P450 member, aromatase.

1.3 Estrogens

Three natural estrogens are produced in vertebrates; namely, estrone (E1; Fig. 1.3, B), E2 (Fig. 1.3, A), and estriol (E3; Fig. 1.3, C). E2 is the most biologically active natural estrogen. Of these estrogens, E1 was the first estrogen to be isolated by Adolf Butenandt and Edward Adelbert Doisy in 1929 followed by the discovery of E2 and E3.[29]
Estrogens are responsible for the development of female secondary sex characteristics (e.g. breasts, wider hips, fat distribution), and for regulation of the menstrual cycle. In women, the major sites for the biosynthesis of estrogens in the ovaries and placenta; and to a lesser extent in the liver, adrenal cortex, fat cells and mammary glands.[30] The biosynthesis of estrogens are increased during pregnancy; this increase is caused by the increased production of estrogens in the placenta.[31]

Estrogens are also important in men; they modulate libido, spermatogenesis, and erectile function.[30] The major site of production of estrogens in men is in the testis by the Leydig cells, but some is also produced in the brain.[32, 33] The levels and roles of estrogens in the body vary depending on the sex, age, stage of fetus development, and pregnancy status of the individual.[34] The levels of estrogens can be altered on purpose by contraceptives; which, in turn, prevents pregnancy.

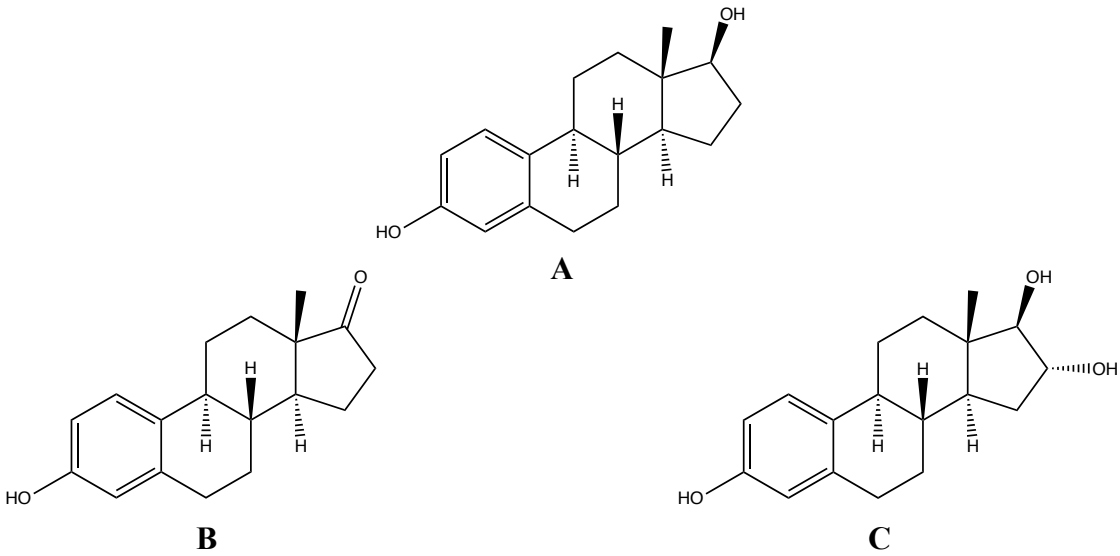


Figure 1.3: Structures of endogenous estrogens E2 (**A**), E1 (**B**), and E3 (**C**).

1.3.1 Oral contraceptives

In the 1960's the oral contraceptive known as "The Pill" was approved for use in the United States of America (USA).[7, 8] Oral contraceptives work achieve this thorough the prevention ovulation by the suppression of gonadotropin release.[35] In these contraceptive, a synthetic estrogen is used, usually 17α-ethinylestradiol (EE2; Fig. 1.4).[7, 8] The structure is strikingly similar to that of E2, sharing the same backbone and similar functional group arrangements. EE2 is an antagonist of ERα and ERβ; which has 233% and 37.8% the binding affinity of E2 for ERα and ERβ respectively.[36] EE2 is the most suitable contraceptive because of the 17α-ethinyl group (Fig. 1.4, A; circled in red). This group prevents oxidation of the 17β hydroxyl by 17β-hydroxysteriod dehydrogenase, keeping it active for longer, thus mediating a higher relative estrogenic activity in the uterus.[37] This means an egg is not released from the ovaries to be fertilised.[37] However, EE2 undergoes rapid metabolism by effective first-pass metabolism which means that very little of the oral dose of EE2 is in circulation un-metabolised.[38] The metabolism of EE2 is typically through conjugation with the addition of a sulfate.[38, 39] This generates the sulfated metabolite, EE2-3-O-sulfate (Fig. 1.4, C).[38, 39] The other metabolism route of EE2 is through hydroxylation, with the main product being EE2-2-hydroxy (Fig. 1.4, B).[38, 39] These metabolites are hormonally inactive and excreted via urine and faeces.[40] Contraception is not the only use for synthetic estrogens, they are also used in the treatment of menopausal symptom.

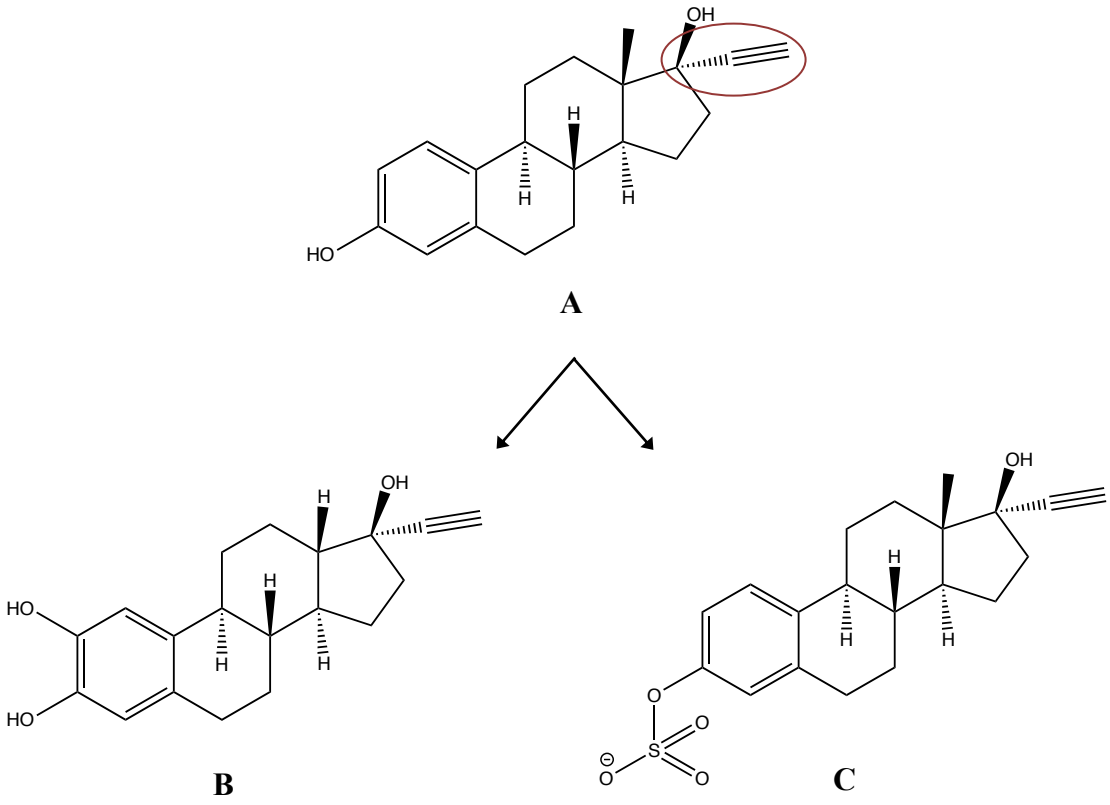


Figure 1.4: The metabolic deactivation of EE2 (**A**) by hydroxylation to produce 2-hydroxy-EE2 (**B**) or by sulfation of the 17-hydroxyl to produce EE2-3-O-sulfate (**C**). The ethyl group is shown circled in red.

1.3.2 Hormone Replacement Therapy

Menopause is the time when menstrual periods stop permanently in women, meaning they no longer are able to bear children. Typically, this occurs between 42 and 59 years of age.[41] Menopause is defined as the decreased production of hormones (e.g. E2) by the ovaries.[42] The decline in E2 levels can lead to symptoms including hot flushes, tiredness, joint and bone pain, and headaches.[41] These symptoms can cause significant disruption at a delicate time in a women's life. These symptoms can be alleviated by the replacement of endogenous estrogens. Compounds such as synthetic mimics of estrogens (e.g. synthetically made E2, progesterone, or progestins, and sometimes, testosterone) are used to replace endogenous estrogens.[9] The replacement of endogenous estrogens with synthetic mimics of endogenous estrogens is known as hormone replacement therapy (HRT).[9] Postmenopausal women taking HRT are shown

to have some symptoms alleviated; for example, fewer bone fractures compared with postmenopausal women not using HRT due to an increased bone density.[43, 44] Another benefit of using HRT is that it can decrease the risk of colon cancer in postmenopausal women.[45, 46] In a study of over 56,000 postmenopausal women, a 17% decrease of colon cancer risk was observed compared with woman who did not use HRT.[47] However, HRT is also associated with negative side effects such as an increased risk of coronary heart disease, stroke, thromboembolic events, and breast cancer with extended use.[9] The risks associated with using HRT to replace endogenous estrogens (e.g. E2) can put doubt in women's minds at this delicate time in their lives because the risk appear large.

1.3.3 17β-Estradiol

E2 is the primary and most potent natural female steroid hormone in humans. E2 is biosynthesized enzymatically in many tissues through an enzymatic pathway. This pathway converts cholesterol to testosterone or E1 and finally to E2. There are many important roles of E2 in humans; for example, it is responsible for secondary sex characteristics (e.g. breast development), puberty onset, and the control of the menstrual and reproductive cycle in females. The level of E2 will depend on the age, sex, and stage of menstrual cycle of an individual.[48-50] The serum E2 concentration in women will fluctuate with progression of the menstrual cycle (Fig. 1.5).[48-50] During the follicular phase of the menstrual cycle, E2 increases, until ovulation is reached, peaking as high as 9 x 10^{-4} mg/L (Fig. 1.5).[51] After the egg is released in the ovulation phase, E2 concentrations decrease and plateau; this is known as the luteal phase (Fig. 1.5).[51] However, E2 levels will fluctuate between the plateau range during this phase (Fig. 1.5).[51] The length of the follicular, ovulation, and luteal phase can vary depending on an individual.

In males, the serum E2 concentration is lower than in women; however, E2 has an important role in sperm maturation and libido. The serum concentration of E2 in males pre-pubescent children, and post-menopausal women range from 5 x 10^{-6} to 4.6 x 10^{-5} mg/L; whereas, a women's serum E2 concentration ranges from 3.1 x 10^{-5} to 4 x 10^{-4} mg/L.[50] Even though the blood concentration of E2 has a large range depending on age, sex, and stage of menstrual cycle, concentrations will be higher than E1 and E3.[48-50]

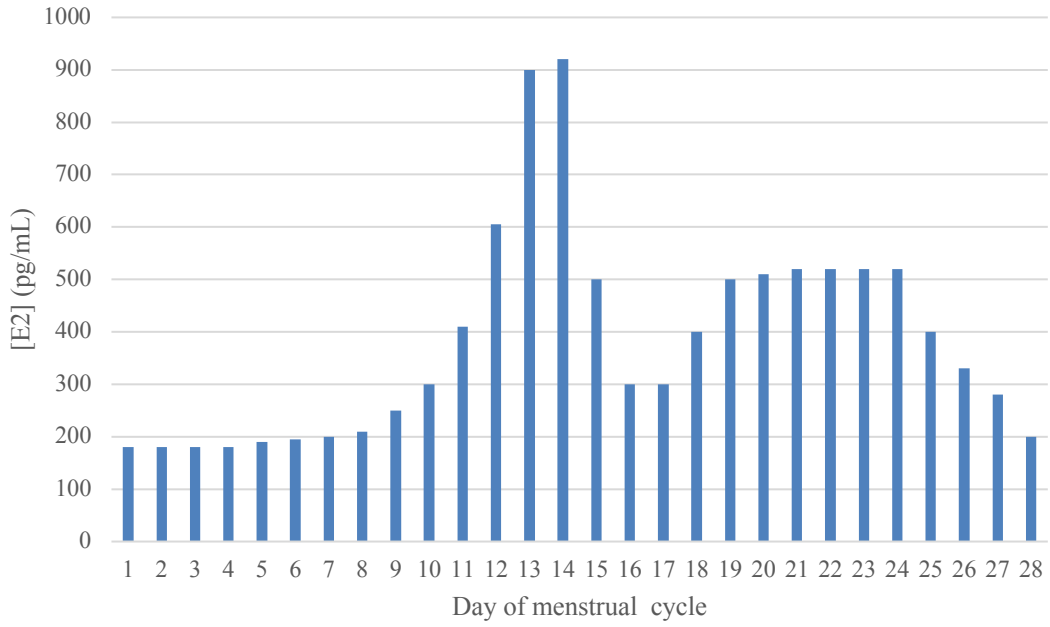


Figure 1.5: Serum levels of circulating E2 in women during the menstrual cycle showing the large fluctuations throughout. Ovulation usually occurs on day 13 or 14. Figure adapted from Shaw IC (2018) Food Safety - The Science of Keeping Food Safe, Wiley-Blackwell, p251, Fig. 9.7.

1.3.4 Estriol

E3 was first isolated in 1930 by Doisy and Marrian from the urine of pregnant women.[52] Soon after E3s discovery, the non-protein bound or 'free' concentration of E3 in the placenta was found to be much higher than E1 and E2 during pregnancy.[53] However, the estrogenic activity of E3 is much lower than E2.[54] During pregnancy, E3 is the most biosynthesised estrogen; with biosynthesis of E3 being increased in the placenta during this time.[55] E3 is also present in high concentrations in human bile, meconium, and the corpus luteum.[56] During the regular menstrual cycle of women, concentrations of E3 are undetectable due to its rapid metabolism and clearance in urine.[55] Unlike E1 and E2, E3 is not biosynthesised in the ovaries, instead it is biosynthesised in the liver. This is achieved through the hydroxylation of E1 and E2 by cytochrome P450 3A4 (Fig. 1.6).[55]

E3 is also used clinically in the treatment of problems associated with a lack of circulating E2 in the body (e.g. during menopause and following removal of ovaries).

E3 treatment works by mimicking the activity of E2 by binding to ERs; and by increasing the concentration of circulating estrogens.

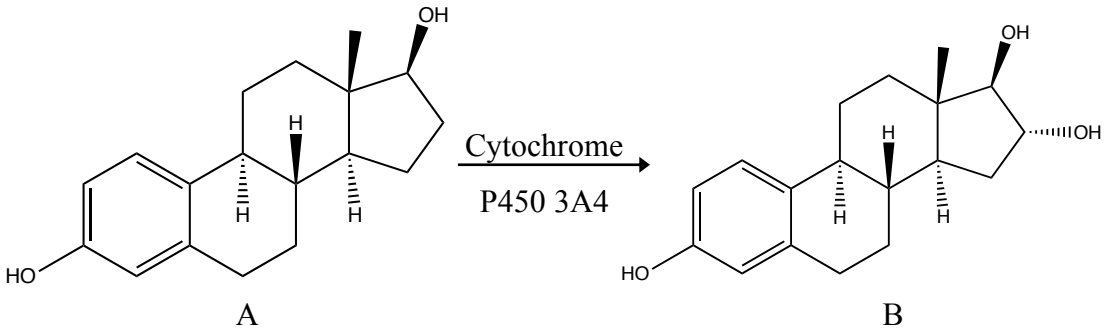


Figure 1.6: Biosynthesis of E3 (B) by hydroxylation of E2 (A) catalysed by cytochrome P450 3A4.

1.3.5 Estrone

E1 was the first pure steroid hormone to be isolated by Adolf Butenandt in 1929 from the urine of pregnant women.[29] Like other estrogens, E1 is biosynthesised from cholesterol in the gonads and adipose tissue. E1 can also be biosynthesised by the reversible conversion of E2 to E1.[37] This conversion is catalysed by the enzyme 17β-hydroxysteroid dehydrogenase (Fig. 1.7) in the liver, uterus, and the mammary glands.[37] Thus, when E2 is required to maintain homeostasis, the cell can carry out the conversion of E1 to E2 to increase the concentration of E2 in the body. This is the likely reason why E1 is used to clinically treat postmenopausal symptoms.

E1 is used to alleviate postmenopausal symptoms such as hot flushes, which is achieved by being converted to E2 in the body, or by mimicking E2 activity through binding to ERs.[37] However, E1 is no longer used in postmenopausal treatment. The reason for this is because synthetic estrogens with better properties, such as, longer activity, oral bioavailability, and higher estrogenic potency were synthesised.

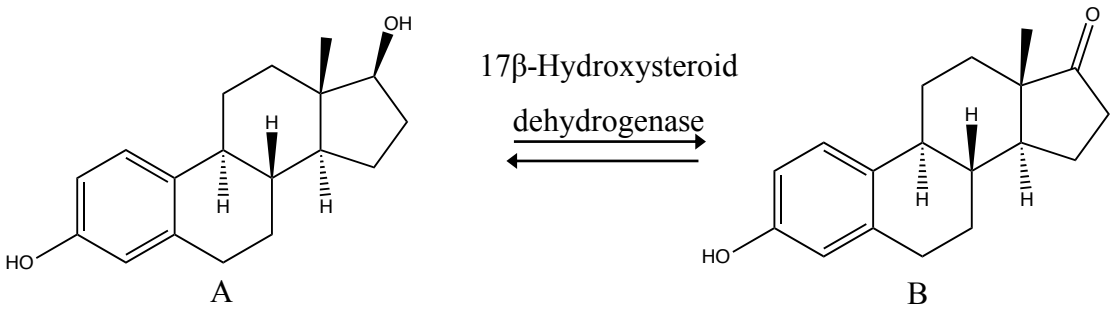


Figure 1.7: The metabolic interconversion of E2 (A) and E1 (B) catalysed by the 17β-hydroxysteroid dehydrogenase enzyme.

1.3.6 Estrogen transport

All steroid hormones are transported bound to carrier proteins in the bloodstream, which increases their water solubility and reduces their activity. A small proportion (1 – 2%) of E2 is present unbound or 'free' from cattier proteins in the bloodstream.[37] This 'free' estrogen is biologically active and able to enter cells to bind to ERs. The remaining 98 – 99% of E2 is bound to one of three carrier proteins; serum albumin, corticosteroid-binding globulin, or sex-hormone binding globulin (SHBG; Fig. 1.8). Binding of estrogens to these carrier proteins renders them inactive, thus the bioavailability of estrogens is governed by the concentration of carrier proteins in the blood stream.[57, 58] SHBG is the most important carrier protein because it has the highest affinity for sex hormones. SHBG has the ability to bind to a specific receptor for the carrier protein on plasma membranes of cells.[57] A allosteric relationship exists between steroid binding and membrane binding.[57] It has been suggested that SHBG receptors exist on sex hormone-dependent tissues (e.g. endometrium, breast) meaning this is a mechanism for controlling the action of sex steroids on their target tissues.[59] Biosynthesis of SHBG occurs primarily in the liver and released into the bloodstream.[60] The biosynthesis of SHBG is decreased in postmenopausal women likely because of the low blood concentration of estrogens, suggesting that SHBG biosynthesis is controlled by the circulating concentration of estrogens.[59, 61] In postmenopausal women, data suggests that there is a correlation between a high risk of breast cancer and high free estrogen levels in combination with low levels of SHBG.[59, 62] This hints that SHBG plays a role in the development of breast cancer. In women who use HRT

the levels of SHBG are reversed because of the increased concentration of estrogens; in turn, this leads to a decreased risk of breast cancer.[59, 61]

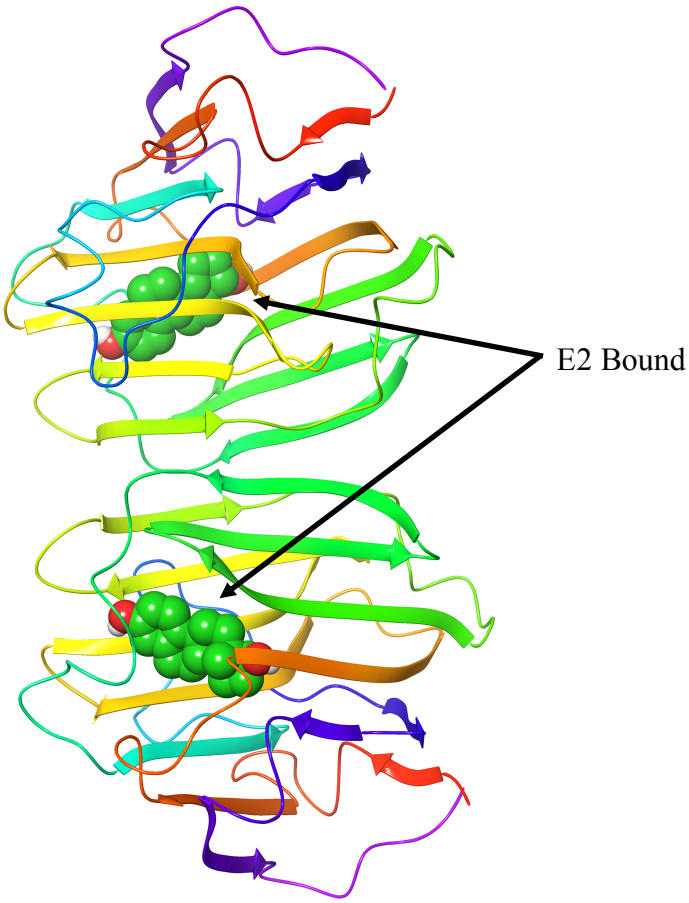


Figure 1.8: Crystal structure of E2 bound to the N-terminal domain of the carrier protein SHBG. Image produced in Schrödinger suite 2017 Maestro. PBD ID: 1LHU[63]

1.4 Estrogen Receptors

Binding of E2 to the ligand binding domain (LBD) facilitates activation, and ligand induced conformational changes of ERs (Fig. 1.9). This conformational change of the ERs exposes amino acids which facilitate dimerisation of ERs.[64] A ER dimer can be a homodimer (i.e. ERα:ERα or ERβ:ERβ), or a heterodimer (i.e. ERα:ERβ). The hormone/receptor dimer complex (Fig. 1.9) is translocated into the nucleus where it binds to specific sections of DNA known as estrogen response elements (EREs),

activating transcription of the estrogen responsive genes (e.g. genes responsible for the synthesis of estrogen).[65]

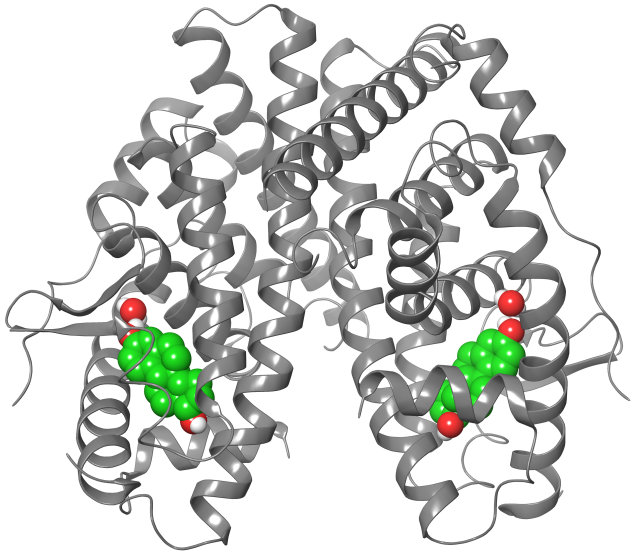


Figure 1.9: Human ERα LBD in complex with E2. Image produced in Schrödinger suite 2017 Maestro. PBD ID: 1ERE [66]

The two ER isoforms are encoded by individual genes located on chromosome 6 and 14 (ERα and ERβ respectively). ERα was discovered by Elwood Jensen in 1962, and described as the estrogen binding protein.[67] Isolation was not achieved until the 1980's when Green *et al.* cloned the DNA encoding ERα present in the MCF-7 breast cancer cell line.[68] A decade later, ERβ was discovered when ERα knockout rats were found to be viable but infertile.[69] It was a surprise that ERα knockout rats were still healthy because of the known importance of E2 to the development of breasts and uterine; as well as the effects E2 has on the cardiovascular system and bone density.[69] This finding suggested that there must be an alternative mechanism by which E2 could affect growth and development (e.g. another receptor).[69] In subsequent studies, ERβ was characterised from a rat prostate cDNA library.[70] The ability for E2 to bind to both receptors suggested that the receptors share a common ancestral receptor gene.[71]

The ERs are evolutionary conserved, with structurally and functionally distinct domains, with the most conserved region being the DNA binding domain (DBD).[70, 72] This region is responsible for DNA recognition and binding to EREs, and shares more

than 95% amino acid homology between ERs.[70, 72] All EREs contain the palindromic hexanucleotide (5'AGGTCAnnnTGACCT3') region where ERs bind to DNA, which is the reason for the large similarity between ER's DBDs.[73, 74] The EREs have an important role in regulation of genes by determining the binding affinity of ERs and modulate the recruitment of co-activators.[75, 76] A ligand bound to the ERs is required before the binding to EREs.

Ligand binding occurs in the –COOH terminal multifunctional LBD.[72] The LBD consists of 12 protein helices which are intimately related; therefore, if there is a conformational change of one helix, this leads to a conformational change of another. When an agonist binds to the LBD of ERs, the 12 helices form a three-layered antiparallel α-helical sandwich. The central core layer comprises H5/6, H9 and H10, with the outer layers comprising of H1 - 4 on one side and H7, H8, and H11 on the other side – which generates a 'wedge shape' structure.[66] These series of conformational changes mediate the actions which are triggered by ligand binding (e.g. ER dimerisation). The LBD protein sequences of ERs has a 55% homology; however, there is high sequence homology between residues 223 – 343 and 404 – 457.[70, 77] These highly conserved regions are the residues which are in direct contact with the ligand, which are highly protected by being buried within the protein structure. The slight differences between the ERs LBDs account for the differences in their affinities for E2.[70] The first X-ray crystal structure of E2 bound to the LBD of ERα was published in 1997.[66] This showed that the LBD comprises 11 α-helices (H1 – H11) and E2 buried in a highly hydrophobic cavity formed by 22 amino acid residues.[66] The hydroxyl groups located at each end of E2 are critical for orientating the ligand in the ligand binding cavity (LBC). The hydroxyl groups of E2 form hydrogen bonds with Glu353 of H3, Arg394 of H5, a water molecule, and His524 of H11 (Fig. 1.10).[66] Other interactions have been suggested to be important for binding; for example, the hydrophobic interactions between the ligand's backbone and the residues in the LBC which are critical for ligand binding and position of the energy necessary for the ensuring conformational changes.[78]

The least conserved region of ERs is the N-terminal domain which varies in sequence and length between isoforms.[70] Transcriptional activation is dependent on two activation function (AF) domains, AF-1 and AF-2. AF-1 is located at the N-terminus and is constitutively active.[72] Whereas, AF-2 is located at the C terminal of the LBD and is ligand dependent.[72] These AF domains recruit a variety of co-regulatory proteins

once ER dimers are bound to EREs. These proteins facilitate activation of the DNA bound receptor/ligand complex. The co-regulatory proteins that are recruited determine the activation of the complex.

ERs are distributed in cells throughout the body; however, there are some differences between their isoforms. ERα has a broad expression pattern, distributed in breast cells, hypothalamus, endometrium, and ovarian stromal cells.[79, 80] Whereas, ERβ has a more focused distribution with high levels found in the ovary, prostate, lung, brain, and endothelial cells.[80, 81] It is possible for compounds which are present in these cells designed to bind to ERs do bind and cause a response.[64]

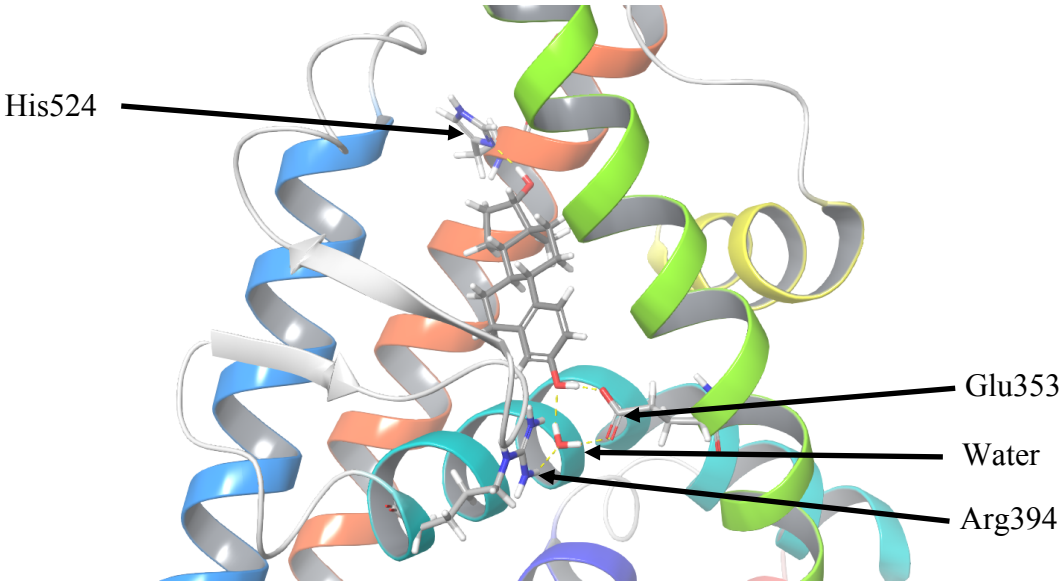


Figure 1.10: X-ray crystal structure of E2 bound to the LBD of the ER. The yellow dashed lines indicate hydrogen bonds. Image produced in Schrödinger suite 2017 Maestro. PDB ID: 1ERE [66]

1.5 Xenoestrogens

Xenoestrogens (from the Greek xenos meaning stranger) are foreign compounds that structurally mimic E2 and are capable of binding to ERs.[64] Over 800 compounds have been identified as xenoestrogens in a recent World Health Organization (WHO) report.[82] Xenoestrogens can be synthetically or naturally produced; synthetic

xenoestrogens are anthropogenic compounds which can contaminate the environment and food; for example, EE2 (from the contraceptive pill; Fig 1.4), bisphenol A (BPA; a polycarbonate plastic monomer; Fig. 1.11), and dichlorodiphenyltrichloroethane (DDT; an insecticide; Fig. 1.11). Natural xenoestrogens are compounds which are naturally produced for example, phytoestrogens produced by plants which are xenoestrogens to humans (e.g. genistein and daidzein from soy; Fig 1.11), and mycotoxins produced by fungi (e.g. zearalenone from mouldy crops stored in humid conditions; Fig 1.11). Xenoestrogens also include pharmaceuticals such as tamoxifen (Fig 1.11) and its metabolite, 4-hydroxytamoxifen (Fig.1.13), used in the treatment and prevention of breast cancer.[83] The xenoestrogens are a concern are because their original purpose was not intended to mimic E2; for example, BPA was designed to be used in the production plastics and epoxy resins such as plastic bottles and sports equipment (e.g. mouth guards) to make them clear and tough. However, BPA has the ability to mimic E2 and cause an estrogenic response by binding to ERs.[84] Over the last century xenoestrogens have been increasingly contaminating the environment due to agricultural runoff, industrial waste, and pharmaceutical use, but long before, plants were producing these estrogen mimics possibly as a way to control the fertility of herbivores.[85]

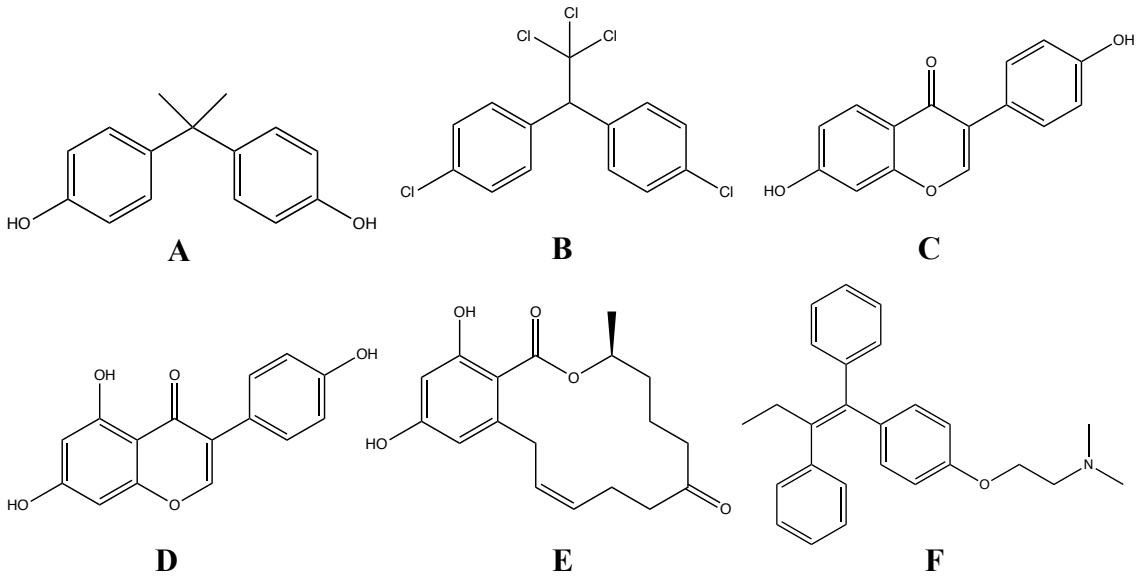


Figure 1.11: Natural and synthetic xenoestrogens; BPA (**A**), a monomer of polycarbonate plastic; DDT (**B**) an insecticide; genistein (**C**) and daidzein (**D**) phytoestrogens from soy; and zearalenone (**E**) from mouldy crops caused by fungi contamination when stored in humid climates; and Tamoxifen (**F**) the pharmaceutical used in the treatment of breast cancer.

1.5.1 Mechanism of Action of Xenoestrogens

A characteristic of entering puberty is the increased production of hypothalamic gonadotropin releasing hormone. In turn, this releases two important hormones from the anterior pituitary gland, luteinizing hormone (LH) and follicle-stimulating hormone (FSH). The response to this in females is for the ovaries to secrete E2, which encourages breast development, fat re-distribution, and skeletal development. Xenoestrogens are able to mimic E2, therefore it is possible that exposure to estrogen mimics before the natural release of E2 could interfere with the process of puberty mentioned previously. This could have effects in the growth and development of children and increase the risk of some cancers (e.g. mammary tumors).[86]

There are two possible mechanisms of actions for xenoestrogens. They can either alter feedback loops in tissues (e.g. gonads, brain, thyroid etc.) by mimicking the estrogenic effects of binding to specific receptors; either activating (agonist) them or blocking (antagonist) them. Alternatively, xenoestrogens can bind to hormone receptors (e.g.

ERs), blocking the natural hormones (e.g. E2) from binding, therefore blocking the natural responses of binding to hormone receptors. Xenoestrogens can be either antagonists or agonists of hormone receptors. If the xenoestrogen is an agonist, it will bind and activate the receptor to produce a biological response (e.g. up regulation of the genes responsible for E2 production). However, if the xenoestrogen is an antagonist, it will block or dampen a biological response by binding and blocking the receptor and prevent or reduce E2's binding. It is possible for a xenoestrogen to be an agonist in one tissue, and an antagonist in another.[87] Most xenoestrogens are weak estrogens, which means that they are partial agonists and/or partial antagonist of ERs.[87] Thus, the estrogenic effects is not completely the effect of the compound, rather, it depends on several factors including the cellular context (e.g. metabolism) and interactions with other signaling pathways.[87] The complete mechanisms of xenoestrogens and how they cause a response are complex and not completely understood.

Xenoestrogens are required to bind to ERs to initiate its mechanism of action.[87] Thus the same or similar binding requirements that exist for endogenous estrogens binding to ERs must be met. The interactions required for binding to ERα are outlined in Section 1.3; briefly, binding to ERα requires two hydrogen bonds to be formed between the ligand and amino acid residues Glu353 of H3, Arg394 of H5, a water molecule, and His524 of H11 of the LBD. An example of a xenoestrogen capable of forming the necessary bonds to facilitate ER binding is genistein (Fig. 1.12). The binding affinity of a ligand depends on the strength of the bonds formed when ligands bind in the LBC, which leads to each xenoestrogen having a different binding affinity for ERs. The binding affinity of xenoestrogens for ERs will likely determine the response that is elicited by ERs. For example, if a compound has weak binding energies with ERα, it might have weak affinity for the receptor. However, if the compound does bind, the conformation change caused by the compound binding is likely to be different to E2; therefore, the response that is elicited is also likely to be different compared with a compound with strong binding energies (e.g. genistein vs. E2). Binding energy probably determines the magnitude of estrogenicity; a compound with large binding energies might cause a conformational change similar to E2, therefore it is likely to be more estrogenic and *vice versa* for a compound with weak binding energies. However,

if the compound binds too strongly, it can inhibit the enzyme (e.g. 4-hydroxytamoxifen).

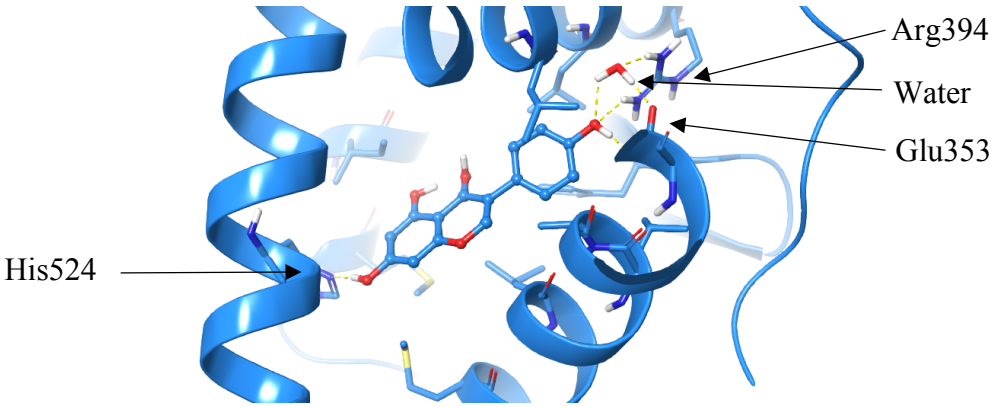


Figure 1.12: Crystal structure of genistein bound to ERα. The yellow dashed lines indicate the hydrogen bonds formed between the ligand and the receptor. Image produced in Schrödinger suite 2017 Maestro. PBD ID:1X7R.[88]

Strong binders of ER can be of helpful in a medicinal context; these compounds are employed in an array of treatment regimens (e.g. to decrease pregnancy complications, in breast cancer treatment) because they block the natural ligand and, therefore, its responses. Diethylstilbestrol (DES; Fig. 1.13, A) is an early example of a medicinal xenoestrogen designed to mimic E2. First synthesized by Leon Golberg in 1938, DES was used for the treatment of breast and prostate cancer, and soon after, prescribed to pregnant women to reduce pregnancy complications and losses.[89, 90] However, in 1971 the USA Food and Drug Administration (FDA) withdrew DES approvals because it was shown to cause a rare endometrial cancer in offspring following *in-utero* exposure. DES is a potent full agonist of both ERs, having 468% and 295% of the affinity of E2 for ERα and ERβ respectively.[77] This strong binding affinity, especially with ERα, is likely the reason for DES leading to endometrial cancers through the disruption of the proper expression of genes regulated by estrogens in the endoemertrium.[91] In ERα knockout mice, complete resistance to chronic effects of neonatal DES exposure was observed compared to wild type mice.[91, 92] However, in ERβ knock out male mice, the same neonatal DES exposure level effects as in wild type mice were observed.[91, 92] This indicates that the agonistic binding of DES to ERα is responsible for the effects in the reproductive tract.[91, 92] Even though the effects of neonatal exposure to DES were

known, DES continued to be used for hormone treatment in advanced breast cancer. This was likely because of its strong binding to ERα, until tamoxifen was approved in 1977. Tamoxifen is a prodrug and is metabolized by two isoforms of cytochrome p450 (CYP2D6, CYP3A4) into the active metabolites 4-hydroxytamoxifen (Fig. 1.13, B), and N-desmethyl-4-hydroxytamoxifen. Both of these metabolites compete with E2 for the ERs active site.[93, 94] These metabolites are ER antagonists, meaning that transcription of estrogen-responsive genes are inhibited, blocking estrogen effects and thus the growth of breast cancer cells.[94] However, not all xenoestrogens have beneficial effects.

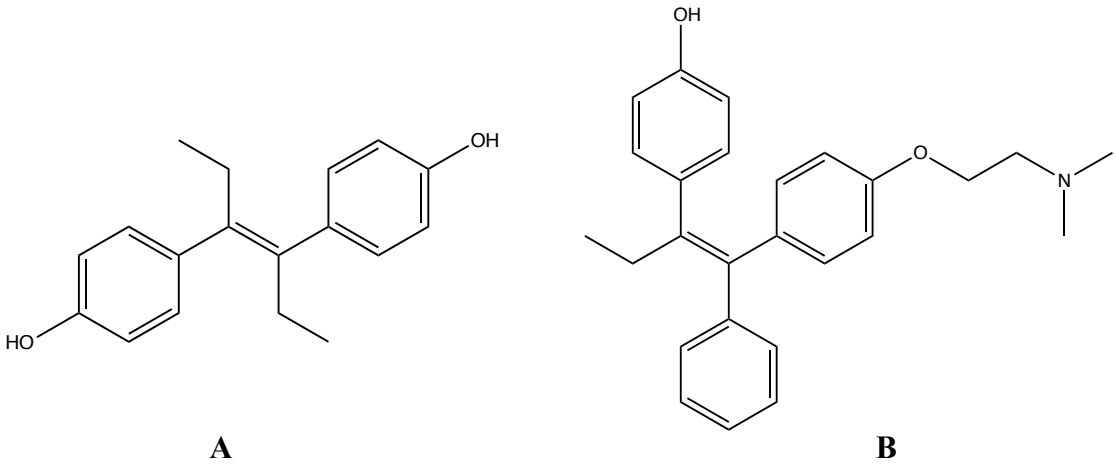


Figure 1.13: Two compounds used in the treatment of breast cancer; DES (**A**) and the active tamoxifen metabolite, 4-hydroxytamoxifen (**B**).

1.5.2 Adverse Effects of Xenoestrogens

For many years xenoestrogens have been associated with many adverse effects in both animals and humans (e.g. intersex fish, gonadal abnormalities in in male alligators).[62, 95-99] These effects are often observed in streams and rivers which are downstream to areas of conurbation (e.g. hospitals, wastewater sewage plants, and agricultural operation). The reason for this is that these areas discharge large amounts of xenoestrogens into rivers. This leads to the aquatic community being highly impacted by the compounds present in the discharge. A good example of this is the effect on fish at sites up stream versus downstream of a wastewater treatment plants (WWTP). Jobling *et al.* showed that the gonadal development of wild roach (*Rutilus rutilus*) living

in United Kingdom rivers receiving large quantities of WWTP effluent presented obvious changes.[100] The changes observed were malformation of germ cells and/or reproductive ducts and altered gamete production, as well as intersex fish with an altered endocrine status and an elevated concentration of plasma vitellogenin.[100] Gonadal growth was only inhibited in severely intersex fish, whereas spermatogenesis was delayed in a large proportion of exposed intersex and male fish.[100] In females living in effluent-contaminated rivers the effects were less obvious, although a higher incidence of ovarian follicle breakdown was identified compared to fish in non-effluent contaminated waters.[100] The plasma E2 concentration of exposed intersex fish were between the concentrations found in control male and control female fish. Whereas, the plasma testosterone concentration was 2 to 3 fold higher in intersex than in male fish.[100] The cause of these changes were a result of exposure to hormonally active contaminants in the WWTP effluent.[100] Vajda *et al.* showed similar effects as Jobling *et al.*, but in white suckers (*Catostomus commersoni*) downstream of a WWTP.[101] It was shown that gonadal intersex, sex ratio, gonad size, ovarian and testicular histopathology, and vitellogenin induction were affected in the white suckers downstream of WWTP.[101] The sex ratio was female dominated at the effluent site with the percentage being 79 - 83% female.[101] Upstream of the effluent site, there was no dominant sex, with the percentage of males being 36 - 46%.[101] Of note, the percentage of intersex fish at the effluent site was 18 - 22%, whereas there was no intersex upstream of the effluent site.[101] These effects are likely to be a consequence of the discharge of xenoestrogens into waterways. Effects of xenoestrogen consumption has also been hypothesised in humans.

In 1992, Carlson and coworkers carried out a meta-analysis of sperm concentrations in men without the history of infertility from two databases.[102] It was found that the mean sperm count had declined from 113 x 10^6/mL to 66 x 10^6/mL from 1940 to 1990.[102] Testicular cancer, cryptorchidism, and hypospadias were also linked decreased sperm counts.[102] It was speculated that the source of these effects were caused by the exposure to estrogen mimics in the environment rather than genetic factors.[102] Since this study, it is still speculated that estrogen mimics have the ability to effect sperm concentration and quality.[95, 102-105] In 2016, a meta-analysis of published phytoestrogen consumption data from the USA and China were compared with their sperm concentrations over a similar time period.[95] The study identified that China's phytoestrogen consumption has declined by 67% from 1991 to 2008, whereas phytoestrogen consumption in the USA

increased by 1613% from 1993 to 2005.[95] These changes in the consumption of phytoestrogens are likely because of the westernisation of the Chinese diet being 'trendy', and the introduction of soy products into the USA.[95] Interestingly, the Chinese sperm concentration increased by 34% from 1999 to 2008, whereas the concentration in the USA declined by 54% from 1938 to 2007 (Fig. 1.14).[95] This suggests a link between phytoestrogen consumption and sperm concentration; a higher phytoestrogen diet might lead to a lower sperm concentration. Xenoestrogens effect the sperm concentrations by having negative effects on feedback loops on the secretion of FSH and LH, affecting the hypothalamo-pituitary system.[103] FSH regulates Sertoli cell replication, this, in turn, coordinates and regulates spermatogenesis.[103] Changes in the quantity and quality of Sertoli cells can occur before and after birth, especially in the pre-pubertal stage which likely affects sperm concentrations in older life.[106] In animals, it has been shown that the alterations in Sertoli cell numbers during early life determines the size of the testicles and sperm output in adulthood.[107] The effects of xenoestrogens are not only limited to developmental problems (e.g. intersex fish, decreased concentration of sperm), it is thought that xenoestrogens can increase the risk of some cancers (e.g. breast cancer)

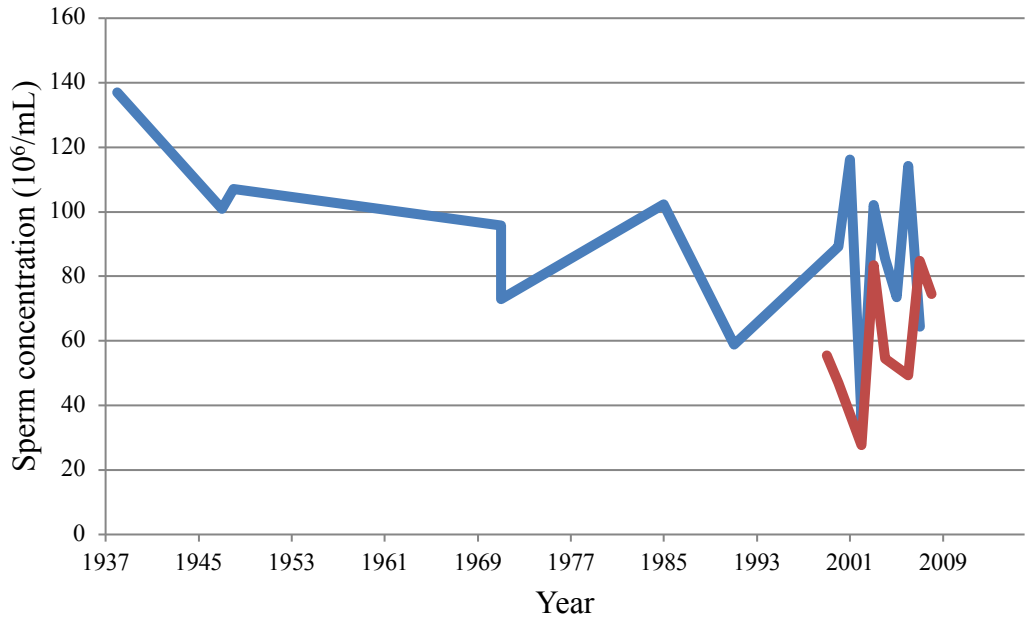
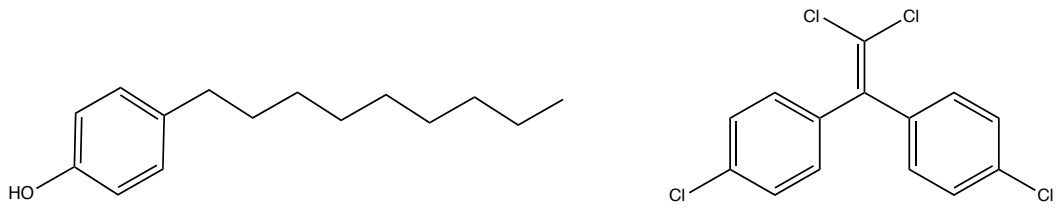


Figure 1.14: Published sperm concentrations for the USA (1938 – 2007) and China (1999 – 2008). Original data from Lim and Shaw (2016).[95]

Breast cancer is the most common cancer in New Zealand women; 3000 people are diagnosed a year, with 600 deaths a year from the disease.[108] Worldwide, breast cancer accounts for 25% of all cancers in women.[109] Of all breast cancers, 83% are ER+, meaning the cancer cells express ERα and grow in response to E2.[110] This means that women who are being exposed to xenoestrogens might have a higher risk of promoting the disease. This might be because of the effects xenoestrogens have on the endocrine system, acting like estrogens or altering the 'normal' metabolism of endogenous estrogens and instead act as carcinogens.[111] The first example of xenoestrogens being able to cause proliferation of breast cancer cells was in 1991.[112] It was demonstrated that proliferation of MCF-7 cells could occur in the absence of estrogens when the media used for culturing was stored in polystyrene centrifuge tubes.[112] If the media was stored in glass centrifuge tubes, E2 supplementation was required for proliferation of these cells.[112] These polystyrene tubes had been modified to make them more resistant to breakage; the compound responsible for the hardening of the plastic tubes, and the proliferation of MCF-7 cells was nonylphenol (NP; Fig. 1.15, A).[112] Following this discover, research on NP continued and more compounds were identified to cause proliferation of MCF-7 cells.[113-116] Soon after these findings, it was shown that the same effect occurs in animals; mice exposed to NP were shown to have a greater risk of breast cancer compared with mice exposed to E2 at the same concentration of NP.[11] The reason for the increased risk is due to NP mimics the actions of E2 through binding to the ERs.[11 117] NP also binds to cell surface ERs, mimicking the signal responses usually controlled by E2.[117] Mice treated with NP had an increased production of E3 in the liver which was believed to be a reason for the increased risk of breast and mammary cancer.[118] However, the primary purpose of xenoestrogens are for a specific purpose and are very useful. For example, BPA is used in the synthesis of polycarbonate plastics and epoxy resins, giving them qualities, such as being clear and tough. Plastics containing BPA are commonly used in bottles, food containers, glasses, teeth fillings, and thermal paper. The benefit that BPA has to offer in the protection of food from contamination is likely to far outweigh the risk of BPA leaching into the food as a xenoestrogen. Xenoestrogens are important because of the benefits they offer.

1.5.3 Exposure to Xenoestrogens

Data on human consumption of xenoestrogens is limited, however, there have been efforts made to estimate the consumption of xenoestrogens.[64] The consumption of xenoestrogens can be assumed based on pesticide usage because they are likely linked. With the population continuing to grow, there is pressure on agriculture to increase production and become more efficient. This leads to the amount of pesticides used to increase because of the continual growth of the population. In developed countries, pesticide usage is monitored, and the pesticides are modified and adapted to be more targeted to the pest, break down faster in the environment, and be less of a risk to humans. This development means that less pesticide is required to be used and is broken down faster, therefore there is less burden on the environment. However, these developments come at a cost; in less developed countries, these modified pesticides are likely to be too expensive. This means that the less developed pesticides will be used which might have been shown to cause adverse effects to humans; for example DDT and the increase breast cancer risk.[119-125] This could explain the link between the amount of pesticides being used, and the incidence of ER+ breast cancers, suggesting that xenoestrogens could be the cause of this link.[126-128] An example of this possible link was shown in wives' of husbands' who sprayed pesticides on fields. These women had an increased risk of breast cancer compared with other women in the cohort study; a hypothesis for the reason this could be happening was because these women in closer proximity to the farms.[128] This also could explain the observation that breast cancer occurrence is higher in lesser-developed countries where estrogenic pesticide usage is higher compared with developed countries.[127]



A **B**

Figure 1.15: Two xenoestrogens known to increase the risk of breast cancer; NP (**A**), and the DDT breakdown product DDE (**B**).

However, phytoestrogens (human xenoestrogens that are produced by plants), are used as an "over the counter" alternative to regular HRT to minimise menopausal symptoms. These symptoms are experienced by 80% of women in menopause, significantly reducing their quality of life.[129] Extended use of prescription HRT used in menopausal women is associated with increased risk of heart disease, stroke, thromboembolic events, and breast cancer.[9] Whereas phytoestrogen alternatives to HRT do not have these risks associated with them. The phytoestrogens (i.e. formononetin, daidzein, and genistein) of particular interest are those derived from soy, and plants of the *Trifolium* (clover) genus; for example, the major components of soy and clover, formononetin, daidzein, and genistein, all have good structural analogies with E2 (Fig. 1.16). In 1998, it was shown that high intake of soy was protective against breast cancer in pre- and post- menopausal women.[130, 131] Phytoestrogen mixes have also been shown to reduce postmenopausal symptoms such as hot flushes and night sweats without the negative effects of HRT (e.g. increased risk of heart disease, stroke, ER rich tissue cancers).[131, 132] Phytoestrogens preferentially bind to ERβ, which may explain the decreased risk of some cancers where ERβ is the primary estrogen receptor (e.g. prostate).[77, 80] Thus, phytoestrogens offer beneficial effects without being associated with the side effects linked to regular prescription HRT.[77] However, it should be considered that genistein, the most prominent phytoestrogen in soy (*Glycine max*) and red clover (*Trifolium pretense)*, enhances proliferation of MCF-7 cells at exposure concentrations as low as 10 nM. Interestingly, genistein can illicit the same effects as E2 in MCF-7 cells at ten times the concentration.[114] Exposure routes to genistein and other phytoestrogens are not just through HRT alternatives; they are very common components of the diet.

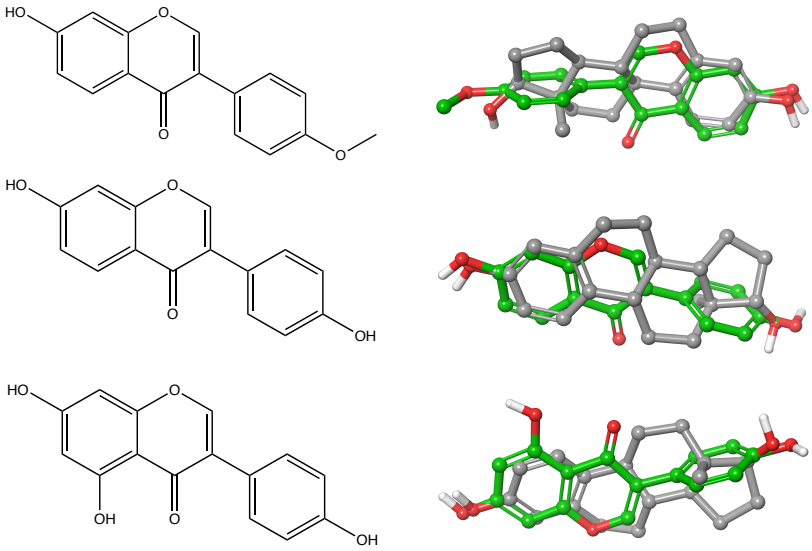


Figure 1.16: The structural similarities can be seen between formononetin (top), daidzein (middle), genistein (bottom), with E2 (dark grey overlay) when superimposed (right respectively). Image produced in Schrödinger suite 2017 Maestro.

1.5.4 Routes of Exposure to Xenoestrogens

Xenoestrogens are present in all areas of daily life; for example, the environment (in its broadest context, including the work place) food and its storage containers, and medicines (Fig. 1.17). The two main categories of xenoestrogens are natural and synthetic. Naturally produced xenoestrogens (e.g. genistein, daidzein) are produced by an organism to possibly allow for the survival of an organism (e.g. defense mechanisms, growth, and signaling). These compounds are not xenoestrogens to organisms producing them and cannot be controlled in food because they naturally occur. Whereas, synthetic xenoestrogens are compounds which were not intended to be estrogenic, unless designed as a pharmaceutical as an estrogen mimic (e.g. for industrial purposes, kill a pest to stop crop ruin, relive pain, or treat a medical condition). As discussed previously, in the case of BPA, the benefits offered by synthetic xenoestrogens are likely to far outweigh the risk of their consumption. Therefore, the risk needs to be assessed relative to the benefits that the xenoestrogen has to offer.

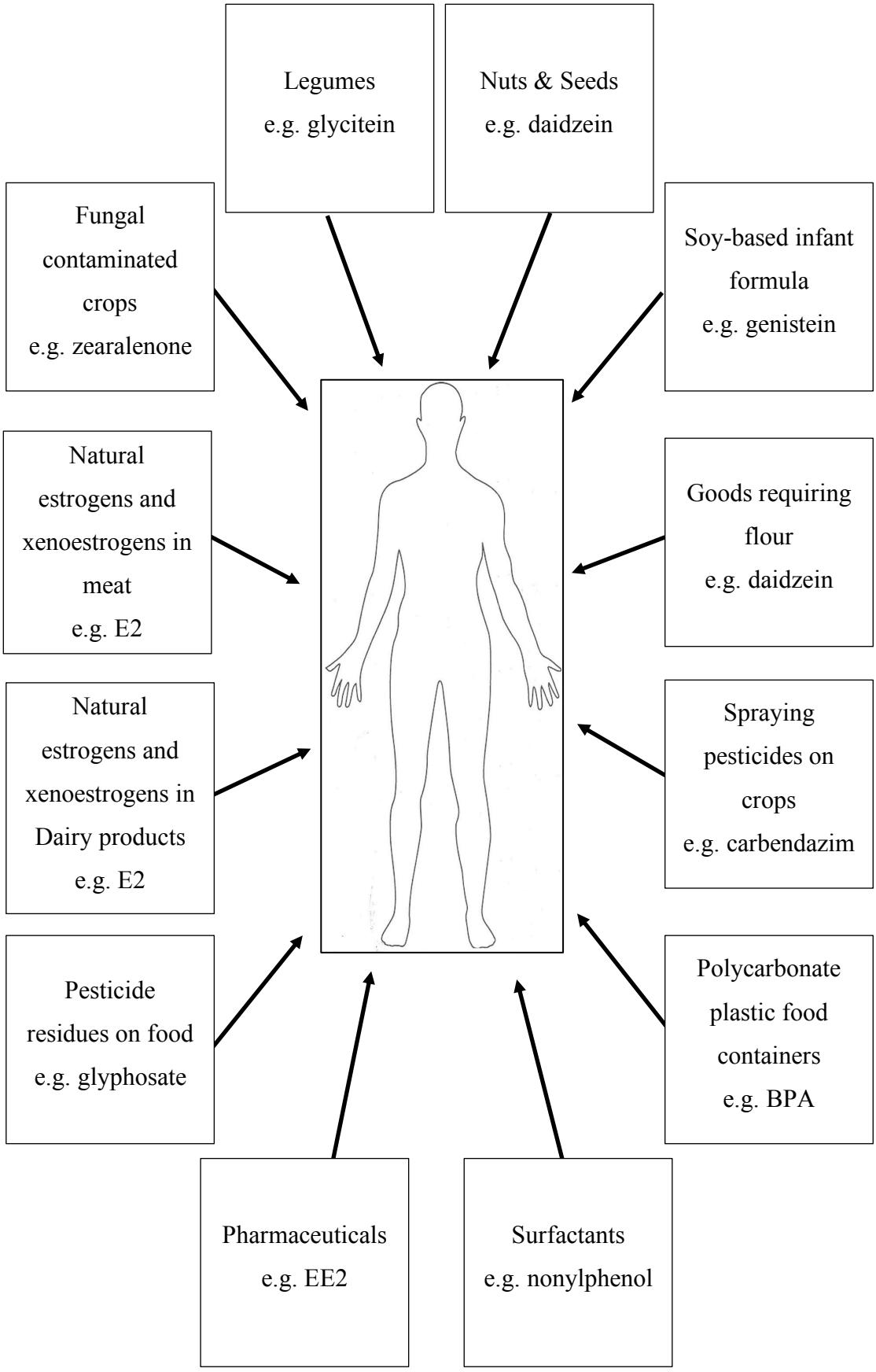


Figure 1.17: The plethora of human exposure to natural estrogen and xenoestrogens in daily life.

Natural xenoestrogens

Natural xenoestrogens are compounds which are not produced by the endocrine system (e.g. genistein to humans). The most common exposure route to natural xenoestrogens is via the diet in the form of phytoestrogens.[133] Typical dietary exposures to phytoestrogens are, but not limited to, soy-based formula, tofu, soy milk, and oats. In New Zealand, products requiring flour (e.g. bread, baked goods, cereal) or products requiring soy lecithin to be made, contain soy. Soy is known to contain phytoestrogens; in particular genistein, daidzein.[133] Therefore, humans have the greatest exposure to genistein and daidzein from the diet. These compounds are not the only phytoestrogens in the diet; lignans and coumestrol are also present. Flaxseed (*Linum usitatissimum*) contains the precursors to lignans which are formed by bacterial fermentation in gut, generating estrogen mimics; for example the bacterial conversion of secoisolariciresinol (found in flaxseed) to enterodiol which bacteria further convert to the estrogen mimic enterolactone (Fig. 1.18).[134] Whereas, coumestrol is an estrogen mimic without metabolism. These are commonly found at high concentrations in pinto beans, soybeans, and black chickpeas (kala chana).[135] Phytoestrogens are not the only natural xenoestrogens which are found in the diet.

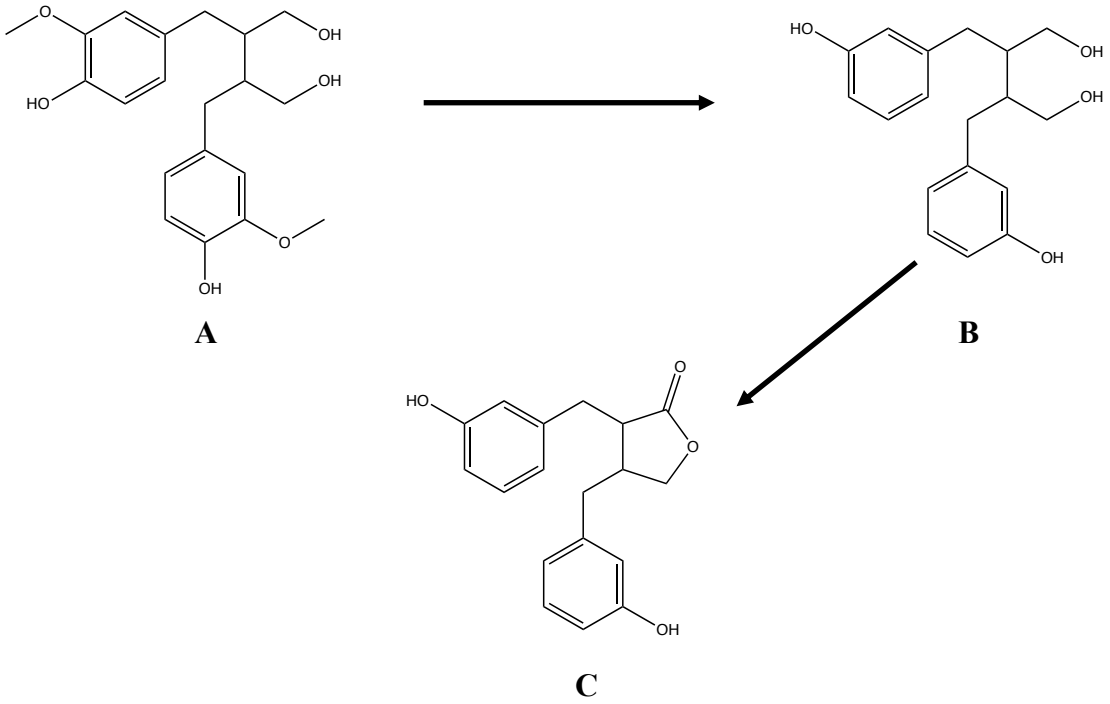


Figure 1.18: The bacterial fermentation in the gut of secoisolariciresinol (**A**) to enterodiol (**B**) and finally converted by the gut microbiota to the lignan, enterolactone (**C**).

Another group of natural xenoestrogens is the mycotoxins. These are toxic substances which are produced by fungus; for example, zearalenone produced by *Fusarium* fungi. Zearalenone is biosynthesized through a polyketide pathway of *Fusarium* fungi. The fungi in the genus which are capable pf producing zearalenone are *F. graminearum, F. crookwellense, F. culmorum*; which are a commonly found in temperate and warm countries.[136] Cereal crops can be grown in these contaminated soils; therefore, these cereals are often contaminated with zearalenone.[136] Mammals who graze these crops or eat these cereals containing zearalenone residues can metabolise it into two stereoisomers (α and β).[137] This is achieved by reducing the C-8 keto group (Fig. 1.19) by the gut microbiota.[137] These metabolites can also be produced by *Fusarium* fungi, but at much lower concentrations than zearalenone.[137] The *Fusarium* fungi grow on and contaminate corn and to a lesser extent oats, wheat, millet, rice, barley, and sorghum and produce zearalenone and its metabolites.[136] Contamination of these crops can also occur after harvesting, due to poor crop management; for example, high moisture environments, poor handling, and not drying the crops. These condition support the

growth of fungi capable of producing zearalenone because they thrive in these conditions.[136]

Endogenous estrogens are also present in the diet, particularly in meat and dairy products.[138] These products are produced by animals which naturally produce endogenous estrogens; therefore these compounds will be present in their meat and milk. When a cow becomes pregnant or be injected with bovine somatotropin (bST), their endogenous estrogen production increases. These are likely to be transferred across the blood-milk barrier because they are important for the development of the calf. Compounds that mimic endogenous estrogens might also be able to cross the blood-milk barrier.

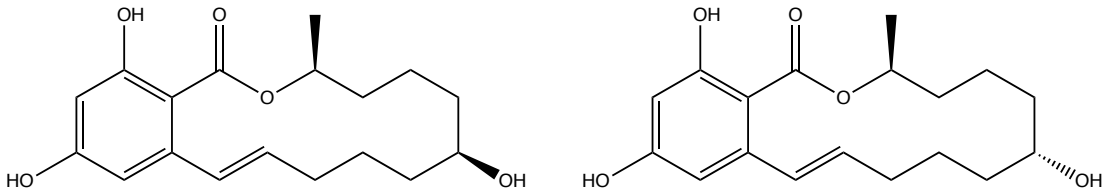


Figure 1.19: Metabolism in mammals of zearalenone by reducing the C-8 keto group into α-zearalenol (**A**) and β-zearalenol (**B**).

Synthetic xenoestrogens

DDT is a known endocrine disruptor and is highly restricted because of this; however, DDT metabolites are some of the most frequently found xenoestrogens in the environment. DDT was one of the first wide spread pesticides, however, today we still live with the long-lasting effects. In 2002, DDT and its metabolites were shown to be present in most food groups in the USA; including, baked goods, fruit, meat, poultry, and dairy products.[139] DDT was banned for agricultural use in the USA in 1972 when it became apparent that DDT was accumulating in the environment, and biomagnifying in organisms. Biomagnification is where the concentration of a compound will increase through the trophic levels until it reaches the top of the food chain. This is where the highest concentration is received (e.g. humans eating a cow that eat grass with DDT residues sprayed on it).[139, 140] DDT and its metabolites are very stable and have low

aqueous stability meaning they persist in the environment.[140] There are very slowly metabolised by microbial degradation, adding to their environmental persistence.[140] Another group of highly persistent estrogen mimics are the polychlorinated biphenyls (PCBs). PCB's are used as coolants and insulating fluids, lubricants, plasticisers in paint and cements, polyvinyl chloride coatings, flame retardants, and adhesives which can be released into the environment. Similar to DDT, PCBs are broken down very slowly by biological transformation meaning they accumulate in the food chain.[139] Thus, they are biomagnified; the properties of PCBs allow their persistence in biological matrices such as human blood, adipose tissue, and milk promoting the biomagnification.[139] Although these compounds were highly used in the past, the burden on the environment is still present, but is decreasing with time. Synthetic estrogens (e.g. EE2) are also commonly found in the environment from industrial effluent and consumer waste. However, they are not as persistent in the environment as DDT and PCBs. The reason for this is likely because they are readily degraded by microbes, plants, light, etc.[141]

The synthetic plasticizer, BPA is commonly used in industry as a monomer in the production of polycarbonate plastics and epoxy resins; used in water bottles, baby's bottle, and the lining of tin cans. It is well documented that BPA can leach from these products into the water and food stored in these containers. Therefore, the potential human exposure to BPA is high.[142, 143] It is unlikely that BPA accumulates in animals because it is rapidly metabolised by glucuronidation followed by excretion via urine.[144]

Other environmental and waterway contaminants include alkylphenolic surfactants; for example, NP and octyphenol. These compounds are used in detergents, fuel and lubricants, polymers, fragrances, antioxidants, oil field chemicals, and fire-retardant materials.[145] NP and octyphenol are known to affect aquatic species downstream from WWTP discharge and agricultural run-off sites.[101] Drinking water has also been found to contain alkylpehenols which is concerning because of endocrine disrupting ability.[146]These compounds are broken down through biological breakdown (i.e. bacteria, fungi) which produce short chain alkylphenols. However, these short chain alkylphenols are persistent due to their poor anaerobic degradation. Under aerobic conditions in the absence of bacteria and fungi, NP is biodegraded into an alcohol and hydroquinone which are not persistent.[145] There is the possibility that these compounds

have the ability to bioaccumulate; however, in rats only doses that exceed the animals detoxification abilities lead to bioaccumulation.[147]

Exposure to these compounds can also come from occupational or clinical exposures. For example, people working in the production of contraceptives and synthetic hormones, or in the production of plastic containers. It is possible that the estrogenic compounds (e.g. EE2, synthetic estrogens, BPA) are absorbed across the skin. This commonly occurs farming; before DDT was restricted, crop dusters would receive huge doses from inhalation and absorption through their skin.[148] People working in these industries are likely to be getting the highest does of xenoestrogens in the population. Exposure to xenoestrogens are broad and not limited to humans, animals are also likely exposed. It is possible that the xenoestrogens that animals are exposed to are passed onto humans through the consumption of their products. Therefore, it is possible that human exposure to xenoestrogens and endogenous estrogens from the diet are the cause of decreasing sperm count, increased incidence of ER+ breast cancer, and earlier age of menarche.

1.6 Milk

Milk is a staple of the western diet, routinely drunk in households in such quantities that it provides a large proportion of the diet. This means that it provides a large source of energy, minerals, vitamins, fats, and 15 other essential nutrients that the body requires.[149] A benefit of milk it that it offers these in an easily digestible and accessible form.[149] The New Zealand population consumption of cow's milk has been consistent over the past decade (Fig. 1.20).[150] In 2013, the New Zealand consumption of cow's milk per capita was 133 mL per day (Fig. 1.20).[150] Interestingly, China's milk consumption has increased by almost 600% over the past three decades, showing the western influence on its diet (Fig. 1.20).[150] A possible reason for this increase is that the western diet has become 'trendy' to consume in China; therefore, increasing the demand of popular western products, such as milk. In New Zealand, milk consumption is encouraged from a young age by schools. Fonterra (the largest dairy co-operative in the world) offers free milk to be given out at primary schools to encourage children to drink milk. Currently, 70% of primary schools in New Zealand have signed up to this

program. Milk consumption in children is import because of the association milk has with better bone and heart health, cancer protection, and decreasing osteoarthritis symptoms due to the calcium and vitamins A and D in milk. However, as mentioned previously, cow's milk is likely to contain endogenous estrogens and xenoestrogens. The reason for this is that cows produce milk pre and post parturition, graze on pasture which might have been sprayed with pesticides and they consume soy-based silage which may have been poorly stored. Xenoestrogens have been shown to cross the blood-milk barrier by passive and active transport; therefore, xenoestrogens from pasture might be present in cow's milk.[12] It is possible that xenoestrogens could also migrate into milk from the polycarbonate containers and epoxy liners.[151] The equipment used during the production process may also be lined with epoxy resins, or made from plastics containing BPA. Thus, it is possible that milk could contain an array of endogenous estrogens and xenoestrogens; therefore, humans who drink cow's milk might receive a dose of these.

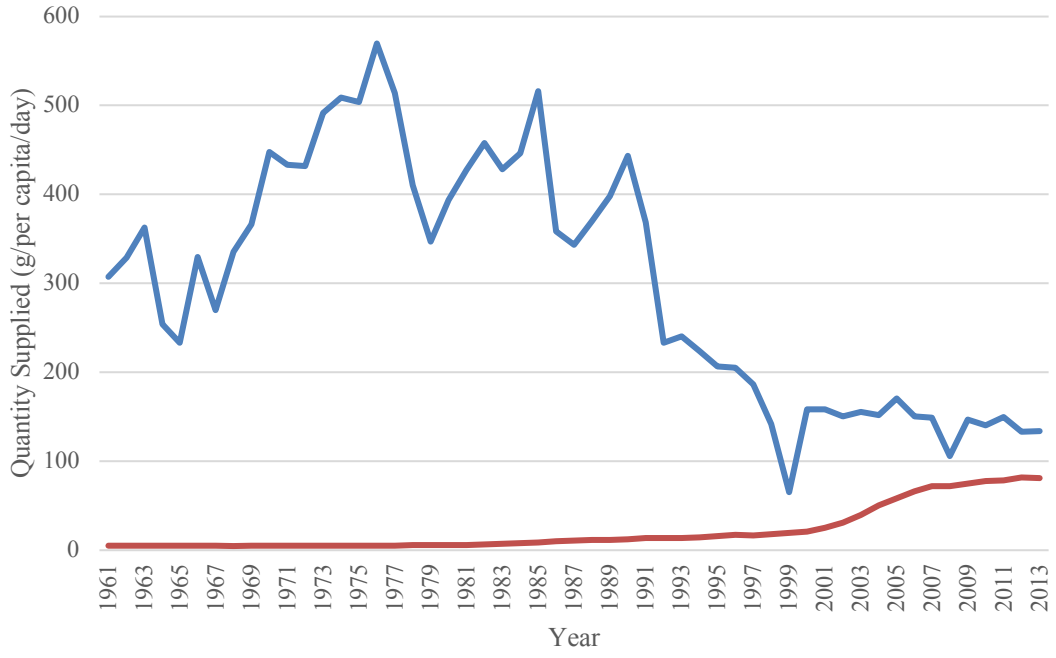


Figure 1.20: Milk supply in New Zealand (blue) and China (red) between 1961 and 2013.[150]

1.6.1 Routes of Milk Contamination by Xenoestrogens

From the cow

Better understanding and technological developments in agriculture have allowed farmers to breed cows which produce higher quantities of milk. Dairy cows must produce one calf a year or be injected with bST and suckled to continue to produce milk. When cows start produce milk, lactation is maintained by constant milking so that milk is continuously produced throughout lactation. Over the last 40 years, the amount of milk a dairy cow can produce has doubled, with the average cow producing more than 26 L/day of milk. 80% of the milk produced is from pregnant cows lactating, thus, the concentration of endogenous estrogens can be high.[152] For example, estrone sulphate (E1S) concentrations can be extremely high in milk from modern cows, with concentrations ranging from 7.9 x 10^{-6} mg/L to 1.26 x 10^{-3} mg/L depending on the cow's stage of pregnancy. E1S is a metabolite of E1 and is biologically inactive. However, E1S has a high bioactivity when consumed orally by humans and can be converted to E1 and E2 in the gut.[103, 153, 154] Therefore, by consuming E1S, it can increase the concentration of active estrogens in the body. The total (free and conjugated) concentration of E2 in cow's milk is much lower, ranging from 1.86 x 10^{-5} mg/L to 5.24 x 10^{-5} mg/L depending on the cow's stage of pregnancy.[12] Although the concentration of E2 is much lower than E1S, but its biological activity is higher than E1S. However, the concentration of endogenous estrogens is likely to be lower than xenoestrogens because of the tight regulations set by regulators.

Farming sources – food and farmer

A cow's food (e.g. grass, silage) and water is likely to be their major exposure route to xenoestrogens. In New Zealand, a dairy cow's primary source of food is pasture (grassland) in paddocks which they graze. This pasture includes red clover (*Trifolium pratense*) and white clover (*Trifolium repens*); both are perennial legumes which are high in protein and fibre. Red clover is short lived and is usually sown in summer in dry areas where grazing is less intense. It does not persist well in high intensive agriculture areas. Whereas, white clover is the opposite; it is grown throughout New

Zealand, persisting well on moderate to high fertility soils, but does not persist well in dry summers. It maintains a high feed quality towards the end of spring when grass plants its seeds. This maintains the milk solid production of dairy cows high. The overall balance of red and white clover depends on the conditions they are in (e.g. time of year, if grazed). Both red and white clover have been shown to contain several phytoestrogens; namely, formononetin, biochanin A, genistein and daidzein.[155-157] These compounds are more concentrated in red clover silage (RCS) compared with white clover silage (WCS), which means that RCS is more estrogenic.[155] RCS was shown to be more estrogenic by feeding cows only RCS or WCS and comparing the estrogenic load of the milk.[155] The milk from cows fed a RCS had a higher phytoestrogen content than cows fed WCS.[155, 156] However, it is possible that formononetin, biochanin A, genistein and daidzein can be metabolised in the cow's rumen by gut microbiota. This results in changed functionality (e.g. the addition of hydroxyl groups, removal of a methyl group) and therefore, a change in their estrogenicity.[155, 157-160] Of note, formononetin can be metabolised to daidzein, which is further metabolized to equol (Fig.1.21).[155, 157-160] This is interesting because equol is more estrogenic than formononetin and daidzein.[158] Thus, it is possible that eqoul is in low concentrations in clover; but after metabolism of formononetin and daidzein by the microbiota in the cow's rumen, the concentration of equol might be higher in milk. Therefore, the estrogenicity of milk would increase. This is important because the metabolites of compounds can be more important in an estrogen mimic context than the parent compounds due to the increased functionality added due to metabolism. It is possible that not all phytoestrogen metabolites have been identified. These metabolites could have a higher estrogenicity than their parent compounds. Therefore, if these are present in cow's milk, it would increase the estrogenic load.

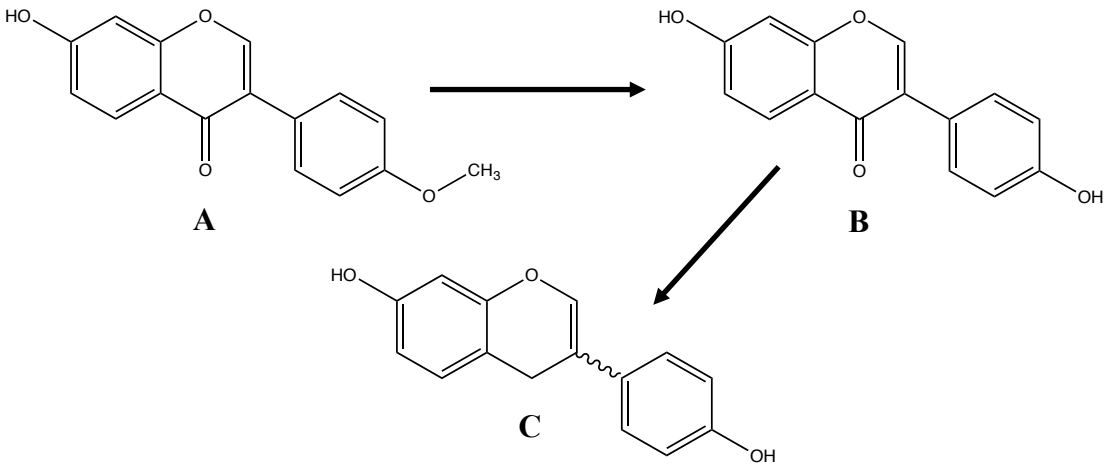


Figure 1.21: Bacterial matablolism of formononetin (**A**) to equol (**C**) via daidzein (**B**) carried out in the rumen of cows.

It is important that pasture quality is maintained to achieve high milk yields in dairy cows. There is pressure on farmer to achieve higher milk yields from cows because the human population is growing. Thus, farmers will protect their pasture which cows graze to maintain the efficiency of their farm. The pressure on farmers to increase production from farms led to the Green Revolution in the 1960s. This aimed to increase agricultural production worldwide by adopting new technologies.[161] The increased efficiency was achieved by planting hybrid crops with increased production, greater acreage, mechanisation, and controlling pests (e.g. insecticides and herbicides).[162] Insecticides and herbicides were adopted because they control pests, weeds, diseases (human, animal, and plant), and increase food production.[163] However, most first generational pesticides were harmful to the environment, with some persisting in soils, aquatic environments, and bio-accumulating in the tissues of animals. The first widely used organic pesticide was DDT; which, led to the development of organophosphates, carbamates, and more organochlorides. Interestingly, one of the first pesticides to be highly restricted was DDT because of the recognition of the hazard DDT had to birds (e.g. increased deaths) and likely to be hazardous to humans (carcinogenicity) with extensive usage.[161, 164] In 2008, a published risk assessment identified 127 pesticides from literature with known endocrine disrupting properties to humans.[165] The most common of these pesticides were insecticides, followed by fungicides and herbicides.[165] The usage of many of these pesticides became highly controlled due to

their negative effects on the environment; however, these compounds still persist in the environment to this day (e.g. DDT).

Glyphosate

The most commonly used herbicide worldwide, glyphosate, is produced by the agrochemical giant, Monsanto. The most common glyphosate-based pesticide is sold under the tradename Roundup. Farmers in New Zealand used Roundup at the end of each season to kill off pasture before new pasture is planted; however, in other countries, such as the USA, farmers use Roundup because 75% of crops have been genetically modified for Roundup. These plants are able withstand high concentrations of glyphosate in their cells without dying, while the weeds die from these high concentrations.[166] The genetically modified crops can withstand high concentrations of glyphosate because they are transfected with a resistant version of the gene for the enzyme targeted by glyphosate, enolpyruvylshikimate-3-phosphate synthase (EPSPS).[167] Glyphosate is absorbed through the plant's roots and transported to growing parts of the plant. Glyphosate kills weeds by inhibiting EPSPS, which catalyes the reaction between shikimate-3-phosphate (S3P) and phosphoenolpyruvate (PEP) to form 5-enolpyruvylshikimate-3-phosphate (EPS3P) through a tetrahedral reaction intermediate (Fig. 1.22).[168] This is a key process in the shikimate pathway, critical for the synthesis of aromatic amino acids (i.e. phenylalanine, tyrosine, and tryptophan), folic acid, lignin, plastoquinones, auxins, phytoalexin, and many other secondary products.[168, 169] Also, over 30% of the plants carbon fixation runs through this pathway.[169] Inhibiting EPSPS with glyphosate leads to deregulated of this pathway, which causes more carbon to be direct through the pathway and the accumulation of shikimate and S3P, diverting energy sources to other areas.[168, 169] Inhibition of EPSPS by glyphosate is achieved by glyphosate occupying the binding site designated for PEP, adjacent to S3P, mimicking an intermediate state of the ternary enzyme-substrate complex. This causes the enzyme to be inhibited because of the increased stability of the enzyme-glyphosate-S3P complex making it inactive.[169, 170]

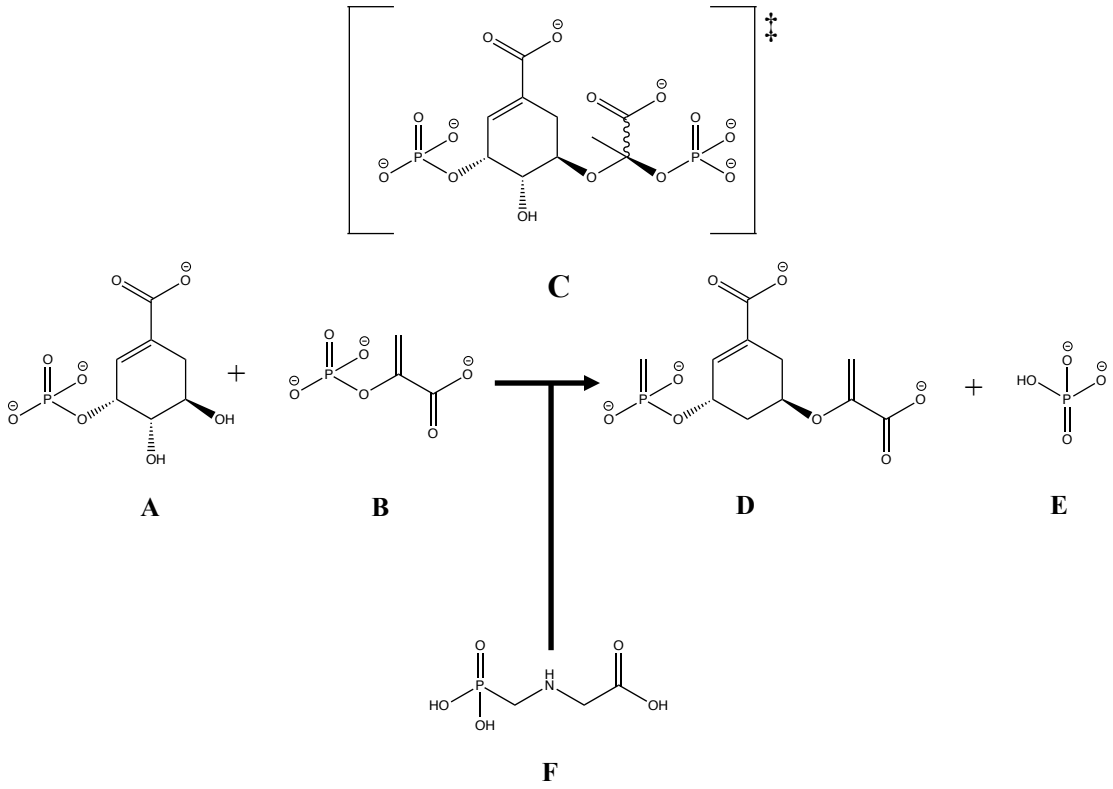


Figure 1.22: The enzymatic conversion of S3P (**A**) and PEP (**B**) to EPS3P (**D**) and phosphate (**E**) through a tetrahedral intermediate (**C**) catalysed by EPSPS. This enzyme is targeted by glyphosate (**F**), mimicking PEP (**B**) and binding to its designated site on EPSPS.

Glyphosate-based herbicides are known pollutants of rivers and surface waters, as well as contaminants of food.[166] It has been shown that shown that these herbicides are toxic to many organisms including human cell lines.[166] Glyphosate is well adsorbed on soils and readily degraded by microbes to aminomethylphosphonic acid (AMPA), which is also strongly adsorbed to soils.[171] However, surface waters are likely contaminated by glyphosate in soil runoff, which is not well broken down by water or sunlight.[171] The half-life of glyphosate is about 47 days in soil, but the soil type and climate can affect its persistence. Whereas, the half-life in water can be up to 97 days. For this reason, pesticides have a withdrawal period, which is the time that products (e.g. pasture) must be left between being exposed to herbicides/pesticides and used or sold. Therefore, it is extremely important that the withdrawal periods set for glyphosate are abided by, especially if the fields are used for grazing and the waters drunk by cattle. If the withdrawal period is not abided by, the cows might receive a dose of glyphosate and

AMPA, which could be transferred to the milk and therefore humans drinking their milk.

Glyphosate toxicity

The toxicity of glyphosate and glyphosate-based herbicides *in-vivo* is still in question likely because the toxicity profile it not understood. A known toxic component of glyphosate based herbicides are the surfactants.[172, 173] These are an important part of these herbicides because they enable glyphosate penetration of the plant's cuticle. [172, 173] Glyphosate was classified as "probably carcinogenic to humans" (category 2A) by the WHO International Agency for Research on Cancer in March, 20015.[174, 175] This classification was based on epidemiological, animal and *in vitro* studies of glyphosate.[174, 175] However, in 2016, a joint meeting between the United Nations Panel of Experts on Pesticide Residues in Food and the Environment and the WHO Core Assessment Group on Pesticide Residues reported that glyphosate was "not associated with genotoxic effects in an overwhelming majority of studies" up to 2000 mg/kg.[176] They declared that glyphosate was "unlikely to be genotoxic at anticipated dietary exposures."[176] This report also stated that glyphosate is unlikely to be carcinogenic in rodents, and it is "unlikely to pose a carcinogenic risk to humans" from dietary exposure.[176] However, studies have shown that glyphosate-based herbicides can cause reproductive effects *in-vivo* (e.g. pregnancy problems, and sperm problems).[166, 177-181] Other effects such as cellular mutagenesis and toxicity have also been shown in cells involved in reproduction (e.g. placental, embryotic and fetal).[166, 182-186] Glyphosate has also been shown to inhibit aromatase (CYP19A1) activity; the enzyme responsible for the conversion of androgens to estrogens (Fig. 1.2).[166] The inhibition of aromatase has been shown to cause intersex gonads and female reproductive issues in amphibians; therefore the same effect could occur in humans.[187] Glyphosate has also been shown to be estrogenic in MCF-7 cells by the proliferation of these cell; these cells grown in response to binding to the ERs.[166, 188] When MCF-7 cells were exposed to glyphosate residues, they proliferated in a dose dependent manner.[166, 188] Binding to ERs was confirmed with the addition of ICI 182, 780 (a potent antagonist of ERs), which blocked the proliferative effects by exposure to glyphosate.[166, 188] This suggests that glyphosate is binding to ERs leading to proliferation of MCF-7 cells.[166, 188] The exposure which caused the half maximal response (IC_{50}) for glyphosate was similar to other

xenoestrogen agonists.[166] Based on the data from human cell lines and animal models, glyphosate might be carcinogenic, mutagenic and reprotoxic at low exposures.[166] Thus, it is possible that glyphosate is a health risk to humans. This contradicts the comments made by the joint meeting between the United Nations Panel of Experts on Pesticide Residues in Food and the Environment and the WHO Core Assessment Group on Pesticide Residues on glyphosate. This meeting commented that glyphosate was not associated with genotoxic effects and unlikely to pose a carcinogenic risk to humans because of the limited studies showing these effects at high concentrations. A possible explanation for the limited studies showing these effects is that glyphosate could be a is a non-genotoxic carcinogen. Therefore, inducing cancer through indirect stimulation of hyperplastic responses, without altering DNA, chromosome number and structure.[189] Non-genotoxic carcinogens have been shown to alter/induce, but are not limited to, endocrine modification (both receptor mediated and non-receptor mediated), immune suppression, tumor promotion, immune suppression, and tissue specific toxicity and inflammatory responses.[189, 190] Non-genotoxic carcinogens have broad modes of action, which are, mostly, tissue and species specific.[189] This could explain the mixed results of glyphosate toxicity in animals models. Non-genotoxic carcinogens are typically negative in tests for carcinogenicity.[189] These tests are usually based on genotoxic endpoints; for example, the AMES test in bacteria, genotoxicity in mammalian cells under *in-vitro* and *in-vivo* conditions, and germ cell mutagenicity tests.[189]

Glyphosate exposure

Glyphosate is used in large quantities in agriculture which could contaminate food products (e.g. cereals). The first food glyphosate was identified in was ice cream from the United Kingdom in 2017.[117] The concentrations that were present were extremely low (0.001 mg/L – 0.00123 mg/L); however, it is possible that these exposures could cause an effect to humans (e.g. reproduction effects, increase the risk of hormone related cancers).[166, 177-186] The concentrations recorded in ice cream are 100-fold lower that the maximum residue limit (MRL) in food other than fruit.[191] This might be concerning because the toxicity profile glyphosate is not well understood. It is possible that the low exposures could cause hormonal effects; for this reason, a lot of controversy surrounds glyphosate. The poor understanding of the toxicity profile has led to significant debates about the legislation around the use of glyphosate and the risk

to consumers. The first ban on glyphosate was in April 2014 by the Netherlands. They passed legislation banning the sale of glyphosate based herbicides for non-commercial use, but allowed industrial use to continue.[192] France followed the Netherlands, asking suppliers of Roundup to be stopped in June, 2015.[193] This was a non-binding request and sales of glyphosate will remain legal until 2022, when home gardening use will be banned.[193] In March 2016, the licensing of glyphosate usage in Europe was due to be renewed, but was stalled due to France, Sweden, and the Netherlands objecting the renewal. Due to this objection, a temporary re-authorization of glyphosate usage was granted in June 2016.[194, 195] This was followed by a license extraction of 18 months.[196] After this period has passed, the majority of the European Union members voted to allow the use of glyphosate to be extended for five more years.

Despite the controversy of the re-licensing of glyphosate in Europe and the unknown toxicity profile of glyphosate, New Zealand would not consider banning glyphosate-based pesticides. The reason for this is because New Zealand's major source of income is from agricultural exports; in particular, dairy. If glyphosate was banned, it would likely have an effect on exports. This is because glyphosate prevents weeds, which increases output of crops by minimising weed competition and space with crops. Thus, not banning glyphosate in New Zealand is understandable because of the benefits it offers to farmers. These benefits likely outweigh the risk glyphosate has on consumers. If glyphosate was banned, there would be a large impact on agriculture because farmers are so reliant on it maintain high outputs. This might also be the reason for no complete ban in Europe. The New Zealand Environmental Protection Agency reviewed the safety of glyphosate in 2006. They employed Dr. Wayne Temple (ex-director of the National Poisons Centre) to review the current data on glyphosate carcinogenicity and suggest any action that should be taken around restricting or banning the use of the compound.[197] Dr. Wayne Temple concluded that glyphosate did not require classification under the Hazardous Substances and New Organisms Regulations as a carcinogen or mutagen.[197] This decision is understandable considering New Zealand farmers reliance on glyphosate-based herbicides to maintain high crop output. However, Dr. Temple's reasoning for the decision to take no action was based on the genotoxicity and carcinogenicity data being of poor quality.[197] This statement is interesting considering the worlds view on glyphosate toxicity; the data's poor quality

and unreliability may be because glyphosate is a non-genotoxic carcinogen (discussed previously), which would be undetected in most systems.

1.6.2 Concentrations of Xenoestrogens in Milk

Chemical contaminants and the nutritional value of New Zealand milk are regularly monitored by the National Chemical Contaminants Programme (NCCP). Milk is the largest export of New Zealand; therefore, the truthfulness of the label is very important. For this reason, the regulations on milk are strict and checked by the NCCP regularly. Monitoring is carried out by the Crown Research Institutes (CRI) which specialise in monitoring chemical components (i.e. ESR). However, the analytical methods used by CRI might not be sensitive enough to monitor compounds below their limits; for example, if glyphosate had an MRL of 0.1 mg/L, and the detection limit of the analytical method used was 0.1 mg/L. This is concerning because xenoestrogens might be able to cause an effect at concentrations lower than the detection and action limits set by the NCCP. Whether or not a compound is monitored will depend on class of compound (i.e. pesticide, phytoestrogen, pharmaceuticals); for example, phytoestrogens will not be monitored because they are natural, therefore it is hard to control their concentration in food. Action limits are toxicity-based measures used by the NCCP; for example, action limits will be based upon acceptable daily intake (ADI) and tolerable daily intake (TDI) concentrations. The ADI and TDI are toxicity based measures of a compound in food and water which can be orally ingested daily over a life time without any adverse health risk to the consumer.[198] An ADI will be used if the compound is intentionally added to food (e.g. food additives, and veterinary drugs and pesticide residues). Whereas, a TDI will be used if the compound is not intentionally added; for example, phytoestrogens from the cow's diet which could be present in milk. There is conjecture around when an ADI or TDI should be used. Compounds can have an ADI and TDI which is the same because they are calculated using the same theoretical principals. These are calculated from the no observable adverse effect level (NOAEL). This is the highest exposure of a compound where no adverse effect is observed (e.g. no change in an enzyme concentration). The NOAEL is divided by a safety factor (usually 100) to get the ADI or TDI of a compound. However, a compound can have more than one NOAEL depending on the stated end point to the toxicity study;

for example, a compound could have an end point of the thickening of the endometrial lining or a change in the concentration of an enzyme in the liver. Depending on the stated endpoint, the NOAEL value can be very different.

Pesticides have their own regulatory limit which is not toxicity based. Instead, it is the maximum concentration of residues which are legally acceptable in or on food or feed when the pesticide is applied correctly; these are called MRLs.

For the purpose of this study, published ADIs will be used. If a compound does not have an ADI, it will be calculated with a hormonal related change as the endpoint where available. It is possible that xenoestrogens will cause a hormonal effect at exposures lower than their ADI. As previously mentioned, an ADI might be calculated from an NOAEL endpoint which is not a hormonal change. It is likely that this alternative change requires a concentration higher than what a hormonal change would require. Hormonal changes are likely to be caused by lower exposures because of the tight regulation of hormone concentrations in humans.

An ADI might be exceeded; however, an ADI is the dose a compound can be received daily for a lifetime with no adverse effects. Therefore, if an ADI dose is exceeded for a short period of time, the risk cannot be assessed. For this reason, the acute reference dose (ARfD) was introduced in 1994. An ARfD is "an estimate of the amount a compound in food and/or drinking water, normally expressed on a body weight basis, that can be ingested in a period of 24 h or less without appreciable health risk to the consumer on the basis of all known facts at the time of the evaluation."[199] ARfD can also be used for slightly longer periods of time such as a week. This value is more useful for regulators because the acute exposure risk can be considered, which allows for a product to be removed from sale if the ARfD is exceeded for a short period of time.

All of these measure (i.e. ADI, TDI, MRL, ARfD) only consider one single compound. However, it is possible that xenoestrogens can be additive – i.e. the cocktail effect. A hypothetical example of this would be if two xenoestrogens were present in a bottle of Milk. In this bottle of milk, xenoestrogen 1 was measured at a concentration of 50 mg/L and xenoestrogen 2 was measured at a concentration of 80 mg/L. If someone drunk 200 mL of this milk, a dose of 10 mg of xenoestrogen 1 and 16 mg of xenoestrogen 2 would

be received. If a 62 kg person consumed this milk, a dose/kg bw of xenoestrogen 1 and xenoestrogen 2 would be 0.16 mg/kg bw and 0.25 mg/kg bw respectively. If the ADIs for xenoestrogen 1 and xenoestrogen 2 were 0.3 mg/kg bw/day and 0.5 mg/kg bw/day respectively for a hormonal change, there would be no risk and legal under legislation. There would be no risk because the dose/kg bw is below the ADI, and therefore legal. However, because there are more than one xenoestrogens present, they are part of part of a cocktail. These compounds are likely to have additive or synergistic effects. If these compounds have structural analogies, they are likely to exert their effects similarly. Therefore, the presence of these two compounds are likely to have a sum of effects of the individual compounds. Synergy is less likely, and complicated to explain. More than one possible mechanism of residues synergy is possible. For example, if a cell was exposed to two compounds (1 and 2) and 1 was toxic, but rapidly detoxified by UDP-glucuronosyltransferases metabolism, and 2 is of low toxicity but inhibits UDP-glucuronosyltransferases, 2 alone will have limited toxicity that is short lived because of the rapid metabolism.[200] However, the situation would be different if both 1 and 2 were administered together.[200] This would result in an enhancement and prolonging of the toxicity of 1.[200] The cocktail effect will, therefore, increase the effects of the individual. This could lead to an increased risk of hormone related cancers and decreased sperm concentration. Those who set regulations are likely to understand this concept but choose to ignore it. The reason for this is because compounds become hard to regulated as it is not completely understood how compounds interact with one another to cause their effect. Overall, the cocktail effect is likely to increase the risk to consumers, even though the individual compounds are below their ADIs.

The concentrations of some endogenous estrogens and xenoestrogens have been measured in cow's milk from different countries. The concentrations of these compounds have big variations from one country to another. In Table 1.1 the concentration ranges of some endogenous estrogens and xenoestrogens from different countries are shown. The ranges can be large; for example, genistein concentrations range from 0.04×10^{-3} to 157×10^{-3} (Table 1.1). The rages of concentrations are likely to be large because of differences in farming methods, weather, and regulations in each country. The growing conditions (e.g. soil fertility, climate) is likely to determine the species which are present in pasture. The growing condition in one country will be best suited to a species high in phytoestrogens better than another. Therefore, the conditions

and species present in pasture is likely to affect the concentration of phytoestrogens in milk. The country which suits the growing conditions of phytoestrogen containing pasture is likely to have higher concentrations in cow's milk.

Regulations from country to country is also likely to be different; in less developed countries, it is possible that the use of hormones in cattle is not illegal. Administering cattle with estrogens have shown to increase their weights, by converting feed into muscle, fat, and other tissues more efficiently.[201] This leads to an increased production of milk per cow and the quantity of meat which can be made from the cattle; therefore, increasing the famers profit.[201] In developed countries, this practice is illegal because of the possible side effects to the milk and meat from the cattle. It is likely that the concentration of hormones in these products would be increased because the increased circulating estrogen concentrations. The estrogens present in this milk and meat would be passed on to the consumer, possible increasing their exposure to estrogens. Some hormones are allowed to be used; in the USA, growth hormones can be given to cattle. The peptide growth hormone, bST, is administered to cows in the USA prolongs the production of milk in cows. bST works by slowing the breakdown rate of cells responsible for producing milk.[202] This is achieved by directing nutrients to these cells extending the milk production period.[202] It is possible that increasing the period of milk production could also increase the concentration of estrogens in the cow's milk. Some countries, including New Zealand, do not allow any hormones to be used in any animals which produce food products (e.g. cow, chicken) because of the possible toxicity to the consumer. However, administering antibiotics to cows is not illegal in most countries. The benefit of antibiotics in cows is so that disease is avoided; therefore, all the cow's energy can be direct towards milk production, increasing the efficiency. However, compounds can still be present in cow's milk without intentionally being added.

A group of estrogen mimics which are typically found in cow's milk are phytoestrogens. These compounds are produced by plants which mimic E2 in humans. The concentration of phytoestrogens in milk will be determine by the cow's diet. This is very hard to control as all cows will eat different amounts. A countries climate is also likely to determine what is in a cow's food; for example, cows in countries with an oceanic climate might have to be fed differently to ones in a tropical climate. The difference in these climates is that an oceanic climate is cool all year round with no wet

season with rainfall evenly distributed throughout the year; whereas, a tropical climate will have a distinct wet and dry season. Therefore, cows in tropical climates may require food supplementation because of droughts in the dry season. Cattles feed is typically supplemented with soy and grains to increase the energy density of the diet. If this is the case, the concentrations of phytoestrogens in milk from these cows are likely to be increased. This is because soy is known to contain genistein and daidzein.[133] Cows in oceanic climates are less likely to require food supplementation because the climate is consistent throughout the year, therefore, can graze pasture continuously. New Zealand has an oceanic climate which is good for the persistent of red and white clover in pasture. As motioned previously, these species of clover contain the phytoestrogens daidzein and formononetin.[155] Pasture containing red and white clover have shown to affect the concentration of phytoestrogens in milk; a cow eating higher concentration of clover lead to a concentration of phytoestrogens in their milk.[155]

The large range of phytoestrogens in milk are shown in Table 1.1, especially daidzein. This is in line with higher clover containing pasture leads to higher concentrations of phytoestrogens in cow's milk. The USA had the lowest daidzein concentration recorded of 2×10^{-5} mg/L. Whereas the higher concentration of daidzein (0.05 mg/L) in cow's milk was recorded in the Czech Republic.[20] Pasture in the USA does not contain high concentrations of clover compared with the Czech Republic, which contains high concentrations of clover.[20] These figures match the thought that clover in pasture affects the concentration of phytoestrogens in milk. In New Zealand, red and white clover are common in pasture. It is likely that the New Zealand conditions are more similar to the Czech Republic. For this reason, it would be expected that the concentration of phytoestrogens in New Zealand are high.

The New Zealand population's daily exposure to endogenous estrogens and xenoestrogens from milk can be estimated. This is based upon the ranges of concentrations presented in Table 1.1, and the daily amount of milk drunk per capita. The most recent data on milk in New Zealand was reported by the Food and Agriculture Organization of the United Nations in 2013.[150] However, this data was for the supply of milk per capita; therefore, for the purpose of this study it will be assumed that the New Zealand population consumes all of the whole liquid milk that it produces. Thus, the amount of whole milk drunk per capita is 132.21 mL per day.[150] The theoretical

daily dose of endogenous estrogens are xenoestrogens are compared with their ADIs in Table 1.1.

The compounds which are closest or exceed their ADIs are the endogenous estrogens; however, New Zealand does not allow hormone use in animals. Despite this, the estimated daily intake of E2 would be 0.03 mg. If a 62 kg person drunk this milk, the dose/kg bw would be 4.8 x 10^{-4} mg/kg bw. E2 has an ADI of 5 x 10^{-4}, therefore the estimated dose/kg exceeds this. If this dose in milk is present for a period of time, it could lead to adverse effects; however, this might not exceed the ARfD of E2.

The other xenoestrogens present in milk (i.e. phytoestrogens, mycotoxins, synthetic estrogen mimics) were measured at concentrations lower than endogenous estrogens. The estimated dose/kg bw was lower than their ADI apart from genistein. For these compounds it is possible that the ADIs set are not based on a hormonal change and instead used an NOAEL value for a change of enzyme concentration. This enzyme change could be an increase in the concentration of aspartate amino transferase. The dose required to cause this enzyme change compared with a hormonal change (e.g. thickening of the uterus lining) is likely to be very different. The dose is likely to be different because circulating hormone concentrations are tightly regulated due to their tight activity range. If there is a slight change in the circulating concentration of hormones, it is possible that an effect will occur. Therefore, two very different ADIs would be produced depending on the endpoint. If an endpoint for an enzyme change is used instead of a hormonal change to calculate the ADI, the consumer could be at risk. However, the relative estrogenic potency (REP) should be considered because these compounds are not as estrogenic as E2. The REP is the estrogenicity of a compound which is usually calculated by measuring a response of a compound binding to ERs (e.g. MELN cells produce light in response to binding to ERα). Phytoestrogens, mycotoxins, and synthetic estrogen mimics do not have the perfect requirements of binding to the LBC of ERs; therefore, the REPs of these compounds are low; for example, the REP of BPA is 5.1 x 10^{-3} % of E2. This means that a higher dose is required to achieve the same response as E2. However, these are calculated for a isolated compound at a time.

As discussed previously, these compounds are likely to be part of a cocktail of estrogenic compounds (e.g. E2 plus daidzein). If the cocktail of compounds is additive upon one another, the dose would be higher. The higher dose received could increase the risk of adverse effects; for example, increase the risk of breast cancer and decrease the concentration of sperm.

New Zealand cow's milk is monitored regularly by ESR; however, the concentrations of endogenous estrogens and xenoestrogens have not been published in literature before. The NCCP publish reports on and comment if compounds were found above a reporting limit, but do not publish the concentration if it is below this. If the concentrations of endogenous estrogens and xenoestrogens in New Zealand cow's milk are similar to those shown in Table 1.1 it could be of concern. Also, these compounds are likely to be present as a cocktail, possibly being additive. Therefore, drinking cow's milk could cause an increased risk of breast, prostate, and testicular cancer, decreased sperm count and sperm quality, and early onset of puberty. Thus, the New Zealand cow's milk risk needs to be assessed by investigating the presence of endogenous estrogens and estrogen mimics.

Table 1.1: Measured concentrations of xenoestrogens in cow's milk and the estimated daily intake based on New Zealand population's milk consumption data. The ADI of the compounds are also included to compared with the theoretical daily intake data.

Compound	**Concentration in milk ($x10^{-3}$ mg/L)**	**Estimated Daily Intake (mg)**	**ADI (mg/kg bw)**	
E2	9 [12] – 253 [13]	1.2×10^{-3} – 0.03	5×10^{-5}	[203]
EE2	0.035 [14] – 0.11 [14]	4.69×10^{-6} – 1.47×10^{-5}	4.3×10^{-5}	[204]
E1	24 [15] – 566 [16]	3.22×10^{-3} – 0.07	8.6×10^{-4}	[204]
Zearalenone	0.046 [17] – 0.76 [18]	6.17×10^{-6} – 1.01×10^{-4}	5×10^{-4}	[203]
Genistein	0.04 [19] – 157 [20]	5.36×10^{-6} – 0.02	1.5×10^{-4}	[205]
BPA	0.47 [21] – 521 [22]	6.39×10^{-5} – 0.06	0.05	[206]
Daidzein	0.02 [19] – 50.2 [20]	2.68×10^{-6} – 6.73×10^{-3}	N/A	

1.7 Research aim

The overall aim of the present study is to assess the risk to consumers of New Zealand cow's milk in an estrogen mimic residue context. This will be achieved by the following research goals:

1) Measure residue levels of estrogen mimics in New Zealand cow's milk
    - Develop a method for the extraction and analysis of a range of natural estrogens and xenoestrogens (E2, daidzein, formononetin, zearalanone, genistein, EE2, BPA, and equol) from cow's milk.
    - Validate the extraction and analytical methods through recovery and limits of detection experiments using high performance liquid chromatography – diode array detector (HPLC-DAD).
    - Assess the applicability of the extraction and analytical methods to cow's milk.
    - Develop a method for the extraction and analysis of glyphosate from cow's milk using liquid chromatography – mass spectrometry (LC – MS)
2) Investigate potential microbiome-mediated increase in estrogenicity of dietary xenoestrogens in a model cow gut system
    - Set up a cow gut model system using fresh cow's rumen tissue samples.
    - Check system to determine whether it is metabolically active (e.g. is equol produced from formononetin?).
    - Investigate microbiota – mediated metabolites of possible milk xenoestrogens using the cow gut model system.
    - Identify any metabolites produced using HPLC-MS, and nuclear magnetic resonance (NMR).
3) Set the xenoestrogens residues levels and cow gut metabolite residues levels in a human risk context.
    - Calculate residues intakes as part of a normal human diet.
    - Compare intakes with ADIs to determine whether ADIs were exceeded.

Chapter 2 – Materials and Methods

Chapter 2 – Materials and Methods

2.1 Materials

2.1.1 Chemicals

Authentic standards

Authentic standards were purchased from Sigma-Aldrich, New Zealand unless stated otherwise.
E2, EE2, BPA, formononetin, zearalenone, daidzein (LC laboratories, USA), genistein (LC laboratories, USA), (*R,S*) – equol (LC laboratories, USA), N-(Phosphonomethyl) glycine (glyphosate).

Solvents

All solvents were HPLC grade and purchased from ECP Ltd, New Zealand unless stated otherwise.
ACN, MeOH.

General Chemicals

All general chemicals were purchased from ECP Ltd, New Zealand unless stated otherwise.
Anhydrous $MgSO_4$, DCM, DMSO glacial acetic acid, hexane, inert N_2 gas, Milli-Q water, NaCl, ethyl acetate, TBME, tris(hydroxymethyl)aminomethane (Sigma-Aldrich, New Zealand), 1M HCl, disodium EDTA (Sigma-Aldrich, New Zealand), ammonium formate, formic acid, Oxid™ Wilkins Chalgren anaerobic broth (Themo Fisher Scientific, New Zealand).

2.1.2 Equipment

Laboratory, measuring cylinder (10 mL, 50 mL, 100 mL, 1L), 50 mL sterile polypropylene (PP) centrifuge tubes, autoclave, automatic pipette and tips (10 µL, 100 µL, 1 mL), C18 reverse phase High Performance Liquid Chromatography column (Phenomenex, New Zealand), Acclaim Trinity Q1 liquid chromatography column (Thermo Fisher Scientific, New Zealand), C18 solid phase extraction (SPE) cartridges (500 mg, 6 mL) (Phenomenex, New Zealand) C18 octadecylsilane (ODS) SPE bulk sorbent (Agilent, USA), centrifuge (Multifuge 1 S-R, Heraeus, Hauna, Germany), glass pasture pipette, glass vial (20 mL), Glass wear (round bottom flasks, beakers, volumetric flasks, separating funnel), High Performance Liquid Chromatography System (Dionex, USA), Rotary evaporator (R-114, and a B-480 water bath, BÜCHI, Sigma Aldrich, Auckland, New Zealand), Schott bottles (150 mL, 200 mL, 500 mL, 1 L, 2 L), 0.45 µM Millipore sterile filter (Merk Millipore, USA). Vortex (DLAB, China)

2.1.3 Biologicals

Sulfatase/β-glucuronidase from *H.pomatia* (≥10,000 units/g solid) (Sigma, USA), Proteinase K from *Tritirachium album* (≥30 units/mg protein) (Sigma-Aldrich, USA).

2.2 Literature Review of the Concentrations of Xenoestrogens in Milk

An extensive literature search was carried out using Google Scholar, Web of Science, Scifinder, and Scopus using the following key words; milk, estrogens, endocrine disruptors, and liquid chromatography, dated from 1990 onwards. From the search results, 21 papers were selected based upon the following selection criteria.

1) Provided concentrations from milk samples.
2) Provided analytical results.
3) Recoveries above 70%.
4) Internal standards.

2.3 Methods

2.3.1 Extraction of Xenoestrogens from Cow's Milk

This section was done in collaboration with Rachel Bennie. All extractions were carried out in duplicate.

Extraction Method 1

The quick, easy, cheap, effective, rugged, safe (QuEChERS) method [207] was applied for extraction of estrogenic compounds from milk. 10 mL of milk purchased from a local supermarket was measured into a 50 mL PP centrifuge tube and 15 mL ACN added. The mixture was shaken for 1 min then 6.0 g $MgSO_4$ added and 1.5 g NaCl and shaken for 1 min. The mixture was centrifuged at 4400 rpm for 15 min. The supernatant was transferred to a 50 mL PP centrifuge tube containing 500 mg C18 sorbent and 1.8 g $MgSO_4$, it was shaken for 1 min then centrifuged at 4400 rpm for 15 min. The supernatant was collected and evaporated at 40°C using a rotary evaporator. The residue was dissolved in 500 µL 50% (v/v) MeOH (aq) and filtered using a 0.45 µm sterile Millipore filter and placed in HPLC vials for analysis.

Extraction Method 2

This method was adapted from Malekinejad *et al.* (2006).[12] Briefly, 10 mL of 80% (v/v, aq) MeOH was added to 10 mL of milk purchased from a local supermarket and vortexed mixed for 10 min then centrifuged at 2000 x g for 10 min at 4°C. The bottom layer was transferred to a 50 mL PP centrifuge tube and mixed with 10 mL hexane and centrifuged at 3500 x g for 10 min at 4°C. The upper hexane layer was removed, and 15 mL DCM added to the remaining liquid, vortexed for 15 sec, followed by slowly shaking by hand for 10 min. Then, the mixture was centrifuged at 2000 x g for 10 min at 4°C. The lower layer was transferred to a 50 mL PP centrifuge tube and the extraction repeated with 15 mL of DCM. The DCM phases were combined and homogonised by vortex. The solvent was evaporated off under a stream of N_2 (g). The

residue was dissolved in 0.5 mL of MeOH and mixed by vortex, followed by the addition 9.5 mL Milli-Q. After homogenisation, the solution was passed through a C18 SPE column (500 mg; 3 mL), which was activated with 5 mL MeOH and pre-conditioned with 5 mL Milli-Q water. The analyte-containing column was washed with 5 mL MilliQ water and dried with a stream of N_2 (g). The analytes were eluted with 4 mL MeOH and dried under a stream of N_2 (g). The dried down particulate material was dissolved in 0.2 mL 50% MeOH (v/v, aq). The solution was filtered through a 0.45 μm sterile Millipore filter and placed in HPLC vials for analysis.

Extraction Method 3

This method was adapted from Wielogorska *et al*.[208] Breifly, 12 mL 1% (v/v, aq) acetic acid in ACN was added to 10 mL of milk purchased form a local supermarket in a 50 mL PP centrifuge tube, followed by the addition 2.5 g of $MgSO_4$ and 1.0 g NaCl. The sample was shaken by hand for 1 min and centrifuged at 4500 rpm for 10 min at 4°C. The supernatant was transferred to a 50 mL PP centrifuge tube and the solvent evaporated at 60°C under a stream on N_2 (g). The sample were dissolved in 5 mL Milli-Q water and passed through a C18 SPE column pre-conditioned with 3 mL of each TBME, MeOH, and MiliQ water. The column was washed with 3 mL Milli-Q water and the analytes eluted with 6 mL of 1% (v/v, aq) acetic acid in 10% (v/v) MeOH in TBME into a tube containing 100 μL DMSO. The solvent was evaporated at 60°C under a stream of N_2 (g) and dissolved in 500 μL 50% (v/v, aq) MeOH and filtered using a 0.45 μm sterile Millpore filter and placed in HPLC vials for analysis.

2.4 Release of Xenoestrogens by De-Conjugation and Enzyme Digestion

2.4.1 Sulfatase/β-Glucuronidase De-Conjugation

Buffer preparation

The buffer was prepared by dissolving 1.64 g sodium acetate in 50 mL of Milli-Q water. The pH was adjusted to 5.0 using 1M HCl. This was made up to 100 mL and making the final concentration 0.2 M sodium acetate.

Incubation

To 10 mL of milk purchased from a local supermarket, 10 mL sulfatase/β-glucuronidase buffer was added to 10 mL of commercially purchased milk and vortexed for 30 sec. 0.5 mg of the enzyme was added to the buffered milk mixture and placed in a shaking incubator at 120 rpm at 37°C. The mixture was then extracted as described in Section 2.3.1, Extraction Method 1. Incubations were carried out in triplicate.

2.4.2 Proteinase K Digestion

Buffer preparation

The buffer was prepared by dissolving 0.36 g tris(hydroxymethyl)aminomethane in 50 mL Milli-Q water. The pH was adjusted to 8.0 using 1M HCl. This was made up to 100 mL and making the final concentration was 30 mM TrisHCl.

Incubation

To 10 mL of milk purchased from a local supermarket, 2 mL proteinase K buffer was added to 10 mL of commercially purchased milk and vortexed for 30 sec. 0.5 mg of the enzyme was added to the buffer milk mixture and placed in a shaking incubator

overnight at 120 rpm at 60°C overnight. The mixture was then extracted as described in Section 2.3.1, Extraction Method 1. Incubations were carried out in triplicate.

2.5 Analytical Methods for the Measurement of Xenoestrogens

2.5.1 HPLC

All samples were analysed using a C_{18} reversed phase column using a stepped gradient. Each run included a solvent only and MeOH only blank. The ultraviolet (UV) absorbance was measured using a photo diode array detector (DAD) at 210, 235, 254, and 280 nm. The sampler was maintained at 4°C and each sample injection was 20 µL. Injections were carried out in dupilicate. The column oven temperature was 30°C.

2.5.2 HPLC Preparation

A Dionex HPLC system was used for all analyses. The injection needle was washed was 10% (v/v, aq) MeOH and primed 5 times with 10% (v/v, aq) MeOH. The HPLC system, was purged for 120 sec with a solvent mixture comprising of 99.9% ACN and 0.1% (v/v, aq) glacial acetic acid, and a mixture of 99.9% Milli-Q water and 0.1% (v/v, aq) glacial acetic acid.

2.5.3 Mobile Phase

Two elution buffers were used: Buffer A; a mixture of 99.9% Milli-Q water and 0.1% (v/v, aq) glacial acetic acid, Buffer B; a mixture of 99.9% ACN and 0.1% (v/v, aq) glacial acetic acid. The same gradient (Table 2.1) was used for all analyses with a flow rate of 1.0 mL/min.

Table 2.1: HPLC gradient for solvent A (a mixture of 99.9% Milli-Q water and 0.1% (v/v, aq) glacial acetic acid) and solvent B (a mixture of 99.9% ACN and 0.1% (v/v, aq) glacial acetic acid)

	0 min	**0 – 20 min**	**20 – 25 min**	**25 – 30 min**
Solvent A (%)	95	50	95	95
Solvent B (%)	5	50	5	5

2.5.4 Identification of Compounds

E2, daidzein, formononetin, zearalanone, genistein, EE2, BPA, and equol authentic standards were run before injection of samples. The sample peaks that co-chromatographed with the authentic standard peaks and matching UV peak maxima were identified as either E2, daidzein, formononetin, zearalanone, genistein, EE2, BPA, and equol.

2.5.5 Calibration Graph Preparation

A 10 mg/L stock solution of analytical standards was prepared by dissolving 1 mg of analytical standard in 100 mL HPLC grade MeOH and 0.1% (v/v, aq) DMSO; it was inverted 20 times to dissolve the compound. The lower concentration standards were prepared by serial dilutions (Table 2.2). This was carried out for E2 (1.0 mg), EE2 (1.0 mg), formononetin (1.2 mg), diadzin (1.3 mg), genistein (1.3 mg), zearalenone (1.2 mg), BPA (1.4 mg) and equol (1.4 mg).

Table 2.2: Preparation of analytical standards

Concentration of Standard	Standard Diluted	Methanol
1.0 mg/L	10 mg/L (10 mL)	90 mL
0.75 mg/L	1.0 mg/L (7.5 mL)	2.5 mL
0.50 mg/L	0.75 mg/L (6.7 mL)	3.3 mL
0.25 mg/L	0.50 mg/L (5 mL)	5 mL
0.10 mg/L	0.25 mg/L (4 mL)	6 mL
0.05 mg/L	0.10 mg/L (5 mL)	5 mL

2.6 Analysis of Glyphosate in Milk

2.6.1 Extraction of Glyphosate from Cow's Milk

The method developed by Chamkasem *et al.* was used to extract glyphosate from milk.[209] Briefly, extracting solvent was prepared by mixing 572 µL glacial acetic acid with 0.74 g Na_2EDTA and made up to 200 mL with Milli-Q water. 3 mL of this solution was added to 1 mL milk purchased from a local supermarket in a 15 mL PP centrifuge tube and shaken by shaking incubator at 120 rpm for 10 min. It was then centrifuged at 3000 rpm for 5 min. 3 mL of the supernatant was passed through a C18 SPE column (500 mg; 3 mL), previously conditioned with 2 mL MeOH and 2 mL extracting solvent. The final 1 mL of the extract was collected into HPLC vials for analysis. This was carried out for 5 milk samples.

2.6.2 Analysis of Glyphosate by LC-MS

Because glyphosate does not have a chromophore, HPLC–DAD detection could not be used. The mobile phase and gradient was applied from Chamkasem *et al.*[209] All mass spectrometry was carried out by Dr. Marie Squire, the School of Physical and Chemical Science (UC)'s technician in charge of instrumentation.

Mobile phase

One elution buffer was used for all analysis. A solution of 500 mM ammonium formate/ formic acid (pH 2.9) was prepared by dissolving 15.76 g of ammonium formate in 300 mL of Milli-Q water. The pH was adjusted to 2.9 with formic acid using a pH meter. This solution was then made up to 500 mL with Milli-Q water. The final LC-MS mobile phase was prepared by mixing 100 mL of the 500 mM buffer solution with 900 mL Milli-Q water, so the final concentration of ammonium formate/formic acid mobile phase was 50 mM.

Instrument method

LC-MS was carried out by Dr. Marie Squire using a Maxis 4G spectrometer operated in high resolution mode with a positive ESI source. The acquisition parameters are shown in Table 2.3 An Acclaim Trinity Q1 LC column with a C18 SecurityGuad guard column was used at 35°C for all analysis. A sample inject volume of 10 μL was used. The flow rate was 0.5 mL/min for a total run time of 6 min.

Table 2.3: The acquisition parameter used for the Maxis 4G spectrometer for glyphosate analysis in New Zealand cow's milk sample extracts.

Scan Begin	100 m/z
Scan End	3000 m/z
Set Capillary	4000 V
Set Nebulizer	1.0 Bar
Set Dry Heater	200°C
Set Dry Gas	8 L/min

2.7 Cow Rumen Metabolism Model System

2.7.1 Sterilisation of Equipment Used for Model Cow Rumen Studies

All glassware, metal instruments and media were autoclaved at 121°C, 15 psi for 20 min.

2.7.2 Media Preparation

Wilkens Chalgren broth was made by dissolving 13.37 g Wilkens Charlgren powder into 405 mL of Milli-Q water. This was then autoclaved as described in Section 2.7.1.

2.7.3 Incubation of Test Substance in the Cow Rumen Model System

Cows rumen samples were obtained from Silver Fern Farms. Slices (4 x 2 cm) of rumen were incubated in 50 mL sterile PP centrifuge tubes containing 15 mL of Wilkins Chalgren anaerobic broth (See Section 2.7.2). To the incubation, 10 mL of formononetin (10 mg/L) was added, and the culture was placed in a shaking incubator at 120 rpm at 37°C for 1, 2 or 3 days in triplicate. Three controls were set up in duplicate in 50 mL sterile PP centrifuge tubes (Table. 2.4) placed in a shaking incubator at 120 rpm at 37°C for 1, 2 or 3 days.

Table 2.4: Preparation of controls for cow's rumen incubation studies

Control	**1**	**2**	**3**
Contents	Wilkins Chalgren anerobic broth only	Wilkins Chalgren anaerobic broth + test compound	Wilkins Chalgren anaerobic broth + rumen slice

2.7.4 Extraction of Rumen Incubate

After incubation, the culture solution was placed in a separating funnel with 45 mL ethyl acetate. The funnel was capped and inverted 15 times and the layers allowed to separate. Once separated, the organic (upper) layer was removed and the solvent evaporated at 50°C using a rotary evaporator. The residue was dissolved in 1 mL 50% (v/v, aq) MeOH and filtered through a 0.45 µm sterile Millipore filter and placed in a HPLC vials for analysis. The HPLC-MS method used as described in Section 2.6.3.

2.8 *In-silico* Molecular Modelling Studies of Glyphosate

The molecular docking studies were carried out using Schrödinger Suite-Small-Molecule Drug Discovery Suite 2017-1 (Schrödinger, LLC, New York, USA) to determine the likelihood that glyphosate would bind to the LBC of the estrogen receptor. Maestro (Schrödinger Release 2017-1: Mastro, Schrödinger LLC, New York, NY, 2017) was used as the interface for preparing ERα and ligands for the study.

2.8.1 Protein Preparation

The X-ray crystallographic coordinates of ERα were taken from the Protein Data Bank (PDB; http://www.rcsb.org), the X-ray crystal structure of ERα complexed with E2 (PDB entry 1ERE) was used. This crystal structure of ERα has four identical chains (i.e. Chain A, Chain B, Chain C, and Chain D), each chain has a docked ligand (i.e. E2). Chain A was arbitrarily used as the ligand-receptor subunit for this docking study, Chain B, Chain C, and Chain D were deleted. Missing amino acid residues (Ser 305, Tyr 331, Asp 332, Pro 333, Thr 334, Arg 335, Pro 336, Phe 337, Arg 548, Leu 549) based on the protein's primary sequence were added to complete the protein structure using the Schrödinger 'Prime' command. Besides the ligand, a single water molecule is present in the protein-ligand complex, this is considered important for ligand-receptor interactions and so was kept, other water molecules (probably originating from the solvent) were removed.[88] A restrained minimization was run to re-orientate side-chain hydroxyl groups and alleviate potential steric clashes by user-selected root-mean-

square deviations (RMSD) with a tolerance of 0.3 Å.

2.8.2 Receptor Grid Generation.

The receptor grid was set up and generated from the Receptor Grid Generation panel of Glide (Schrödinger Release 2017-1: Glide, Schrödinger LLC, New York, NY, 2017). The natural ligand binding site was used for this docking study, but the original bound ligand (E2) was excluded.

2.8.3 Preparation of Ligands

Glyphosate was built and prepared using LigPrep to set the ionization state at pH 7.0 ± 2 and generate possible tautomers. (Schrödinger Release 2017-1: LigPrep, Schrödinger LLC, New York, NY, 2017).

2.8.4 Ligand Docking and Calculations

Rigid receptor-flexible ligand docking calculations were performed using Glide in extra precision (XP) mode. Glyphosate was conformationally sampled in the LBC and its pose was scored in terms of its E-model. Since the E-model has a more significant weighting of the force field (electrostatic and Van der Waals energies), it is well-suited to pose selection. In this docking experiment, GlideScore and DockScore were determined. GlideScore approximates ligand binding free energy, it has many components, including force field (electrostatic, Van der Waals) contributions and factors rewarding or penalizing interactions known to influence ligand binding. The resulting docking poses were ranked using DockScore which is the sum of the GlideScore and the Epik (i.e. state penalty for ligand protonation and tautomeric). The hydrogen bonds and π-π interaction were shown using a Ligand Interaction Diagram.

Chapter 3 – Results

Chapter 3 – Results

3.1 Concentrations of Endogenous Estrogens and Xenoestrogens in Milk

Searching the key databases with the selected key words, resulted in 21 references based on the selection criteria listed in Section 2.2. The compounds that have been measured to date in bovine milk are the natural estrogens (E1, E2, E3, and E1S), phytoestrogens (formononetin, equol, biochanin A, daidzein, and genistein), mycotoxins (zearalenone and its metabolite α-zearalenone), and estrogen mimics (EE2, BPA, NP, and octylphenol). The concentrations differ between countries and type of milk. Table 3.1 shows the concentrations of estrogens and estrogen mimics, the country, and the reported concentration in specific milk types. Table 3.2 shows the reported concentrations of estrogens and estrogen mimics in milk which did not specify the type.

Table 3.1: Published concentrations of endogenous estrogens and xenoestrogens in specific types of cow's milk (organic [O], raw [R], whole [W], half skim [HS], and skim [S]) from different countries.

Compound	Country	Reported concentration (mg/L) $x10^{-4}$
E1	Iran	2.22 [O][12]
		0.93 [R][12]
		1.62 [W][12]
		1.74 [W][12]
		2.08 [W][12]
		2.43 [W][12]
	Spain	0.25 [R][14]
		0.75 [R][14]
		0.80 [R][14]
		0.35 [W][14]
		1.30 [W][14]
		35 [W][14]
		12 [HS][14]
		0.45 [S][14]

E1 cont.		0.60 [S][14]
	United States	0.10 [W][15]
		0.84 [W][210]
		0.84 [HS][210]
		0.65 [S][210]
	Germany	1.30 [W][210]
		55.10 [S][211]
	France	1.87 [W][212]
		1.41 [HS][212]
		2.07 [S][212]
E2	Iran	0.12 [O][12]
		0.11 [R][12]
		0.09 [W][12]
		0.10 [W][12]
		0.10 [W][12]
		0.11 [W][12]
	Spain	0.85 [R][14]
		31 [R][14]
		32 [R][14]
		11 [W][14]
		12 [W][14]
		54 [W][14]
		23 [HS][14]
		11 [S][14]
		12 [S][14]
	France	0.135 [W][212]
		0.13 [HS][212]
		0.166 [S][212]
	United States	0.22 [W][210]
		0.22 [HS][210]
		0.20 [S][210]
	Germany	26.6 [S][211]
E3	Iran	0.12 [O][12]

E3 cont.		0.1 [W][12]
	United States	0.03 [W][210] 0.01 [HS][210] 0.01 [S][210] 1 [S][210]
E1S	United States	0.85 [W] [15]
EE2	Spain	0.20 [W][14] 0.35 [W][14] 1.10 [W][14] 0.25 [S][14]
Formononetin	Finland	45 [O][158]
Equol	Finland	4100 [O][158]
	Czech Republic	30 [R][213] 30 [R][213] 40 [R][213]
	Australia	450 [R][157] 610 [R][157] 680 [R][157] 680 [R][157] 2930 [R][157]
Daidzein	Czech Republic	160 [R][213] 140 [R][213] 110 [R][213] 100 [R][213]
Genistein	Australia	40 [R] [157] 220 [R][157] 250 [R][157] 290 [R][157]
	Czech Republic	160 [R][213] 160 [R][213] 510 [R][213] 570 [R][213]
Zearalenone	China	0.01 [R][17]

α-Zearalenone	China	0.02 [R][17]
BPA	Spain	9.90 [W][214]
		11.70 [W][214]
		12.90 [W][214]
		26.40 [W][214]
	Italy	150 [W][21]
		150 [W][21]
		170 [W][21]
		360 [W][21]
		450 [W][21]
		481 [W][21]
		150 [S][21]
		160 [S][21]
		620 [S][21]
		730 [S][21]
		880 [S][21]
		1700 [S][21]
		5210 [S][21]
NP	Taiwan	38 [W][215]
		44 [W][215]
		57 [W][215]
		80 [W][215]
		36 [HS][215]
		50 [HS][215]
		36 [S][215]
	Spain	165 [W][214]
		236 [W][214]
		277 [W][214]
		348 [W][214]

Table 3.2: Published concentrations of endogenous estrogens and xenoestrogens in un-specified types of cow's milk from different countries.

Compound	Country	Reported concentration (mg/L) $x10^{-4}$
E1	China	0.50[216]
		0.70[216]
	France	1.52[217]
E2	China	12.50[218]
	France	0.23[217]
E3	China	1.20[216]
		1.40[216]
		2.30[216]
		21.30[219]
		61[218]
Formononetin	France	12[220]
Equol	Finland	616[158]
	France	780[220]
Biochanin A	France	7[220]
Daidzein	France	18[220]
	United States	0.24[19]
Genistein	France	9[220]
	United States	0.35[19]
Zearalenone	China	205[17]
α-Zearalenone	China	0.03[17]
BPA	China	4.90[22]
NP	China	42.40[22]
		53.80[22]
		55[22]
		76.80[22]
		112.90[22]
		159.30[22]
		176[22]
Octylphenol	China	1[22]
		1[22]

Octylphenol cont.	1[22]

3.2 Identification of Compounds

A peak's identity in milk sample extracts was confirmed by comparison with retention times (t_R) of authentic standards and its UV-spectrum. These were compared with authentic standards that had been purchased. A comparison for a BPA authentic standard and a suspected BPA peak present in milk sample extract is shown in Figs. 3.1 and 3.2 respectively. Detection of all compounds was at the wavelength λ 280 nm.

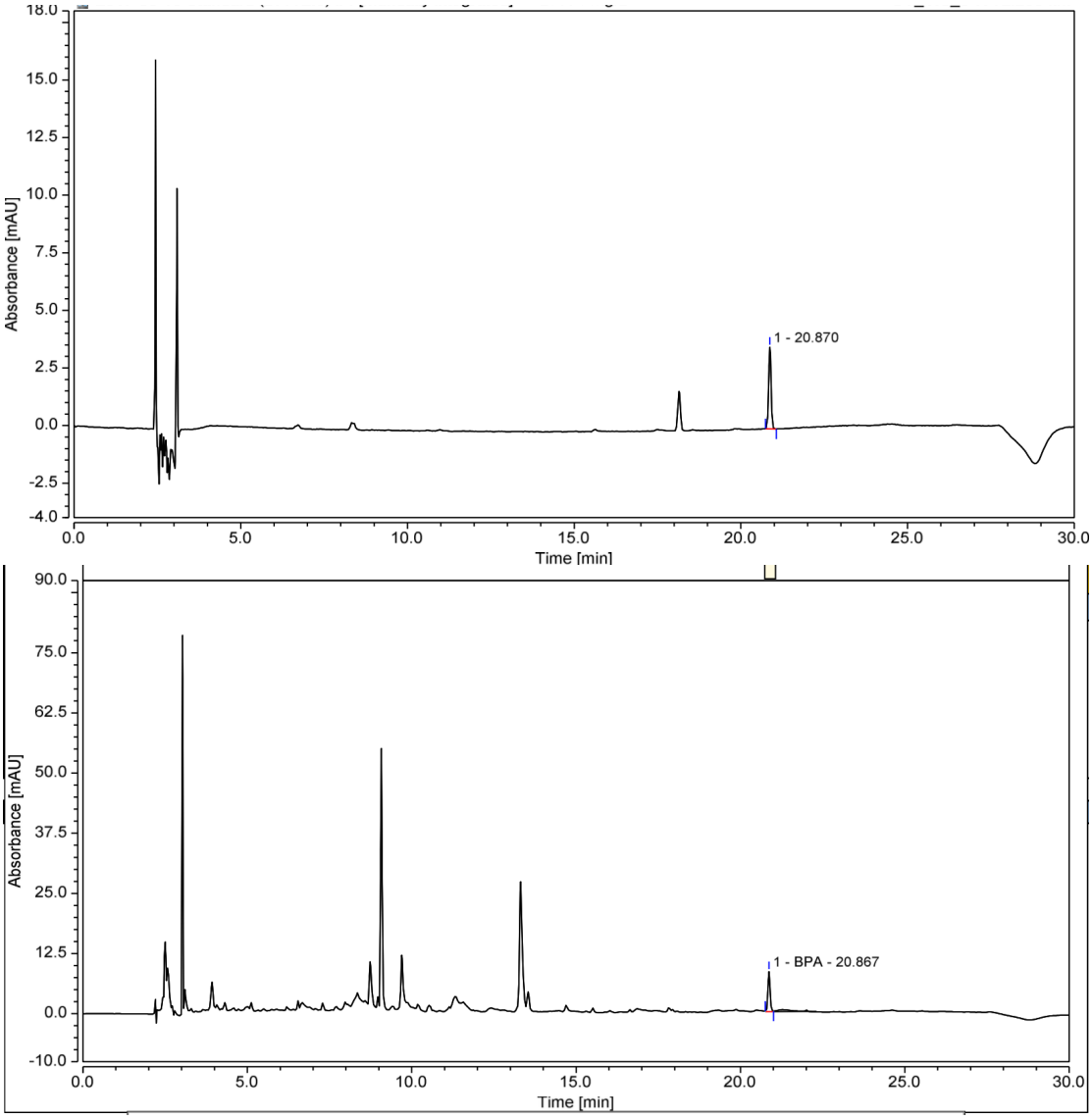


Figure 3.1: A typical HPLC trace for BPA authentic standard (t_R = 20.870 min) (1 mg/L in ACN) (top) and a suspected BPA peak (t_R= 20.867 min) in a milk sample extract injection. Detection at λ 280 nm.

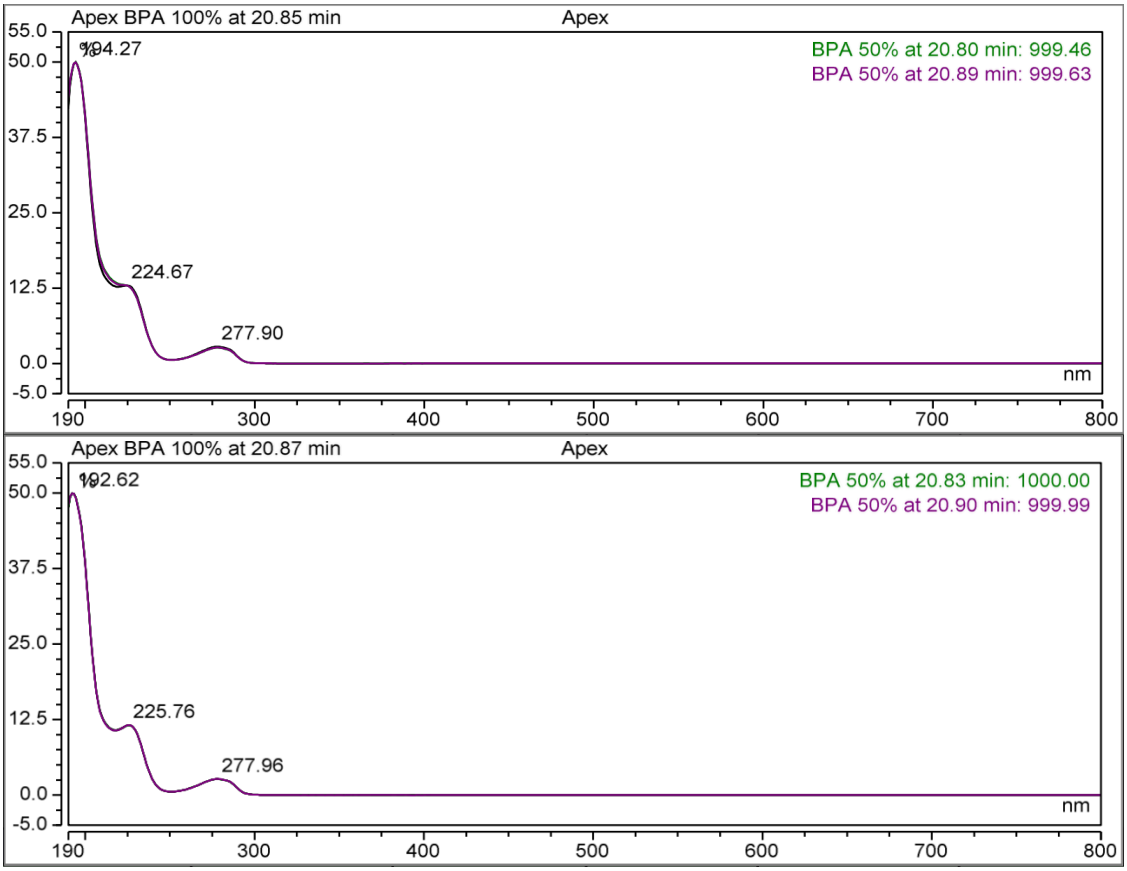


Figure 3.2: Typical UV spectra of BPA authentic standard (1mg/L in ACN) (top) and a suspected BPA peak in a milk sample extract (bottom) injections using HPLC-DAD.

3.3 Validation of the HPLC-DAD analytical method for Endogenous Estrogens and Xenoestrogens

The separation of E2 and xenoestrogens (daidzein, genistein, formononetin, BPA, EE2, and zearalenone) was successful using the HPLC-DAD method described in Section 2.5 (Fig. 3.3). The peaks were well separated and were sharp with no shoulders (Fig. 3.3). All the compounds studied were eluted within 10 min (Fig. 3.3 and Table 3.3).

Table 3.3: The HPLC retention times of E2 and xenoestrogens (for solvent systems see Section 2.5.3)

Compound	t_R (min)
E2	21.860
EE2	23.060
Formononetin	20.530
Genistein	18.533
Daidzein	15.617
Equol	18.600
BPA	20.867
Zearalenone	24.697

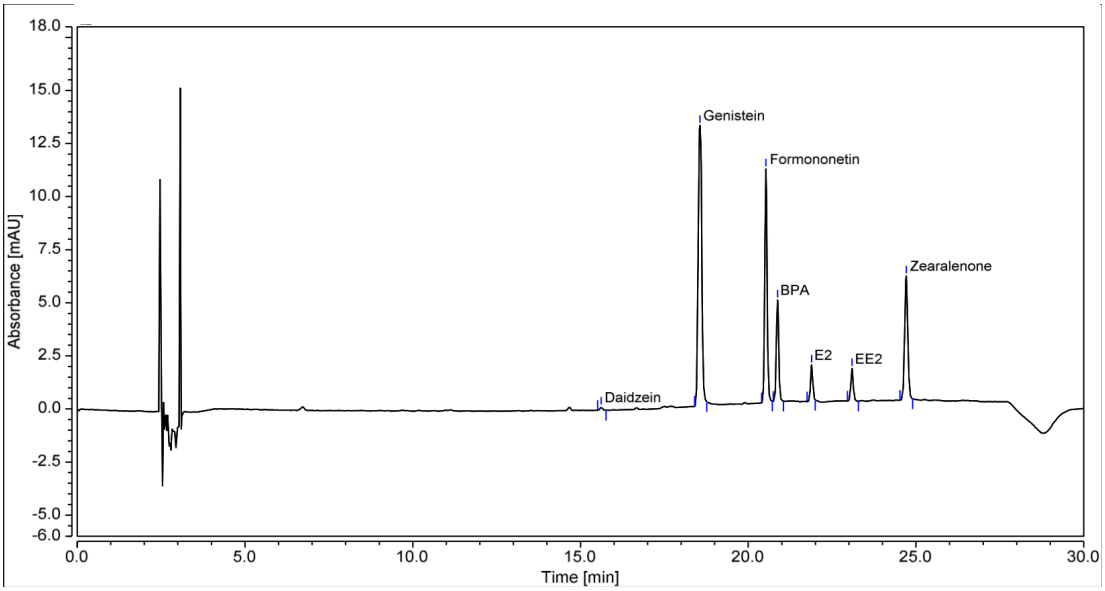


Figure 3.3: An HPLC trace for a solution of 50% MeOH (v/v, aq) containing daidzein, genistein, formononetin, BPA, E2, EE2 and zearalenone authentic standards injection. Authentic standards separated within 10 min. Detection at λ 280 nm.

3.4 HPLC-DAD Limit of Determination and Limit of Quantification for Xenoestrogens

Method detection and quantification limits are presented in Table 3.4. The limit of determination (LOD) values for E2, EE2, formononetin, genistein, daidzein, equol, and

BPA were measured by the sound to noise ratio of two MeOH blanks. The LODs ranged from 0.0025 – 0.1 mg/L for E2, EE2, formononetin, genistein, daidzein, equol, and BPA. The limit of quantification (LOQ) being at least 10-fold more concentrated that the LOD, ranging from 0.05 – 1 mg/L for E2 and xenoestrogens. A typical chromatogram of a sample blank, HPLC grade MeOH (Fig. 3.4), and the detection limit and quantification limit chromatogram of daidzein is shown in Figs 3.5 and 3.6 respectively.

Table 3.4: LOD and LOQ for E2, EE2, formononetin, genistein, daidzein, equol, and BPA using HPLC-UV.

Compound	**LOD (mg/L)**	**LOQ (mg/L)**
E2	0.1000	1.00
EE2	0.0500	0.10
Formononetin	0.0050	0.05
Genistein	0.0050	0.10
Daidzein	0.0025	0.05
Equol	0.0100	0.05
BPA	0.0050	0.01
Zearalenone	0.0250	0.05

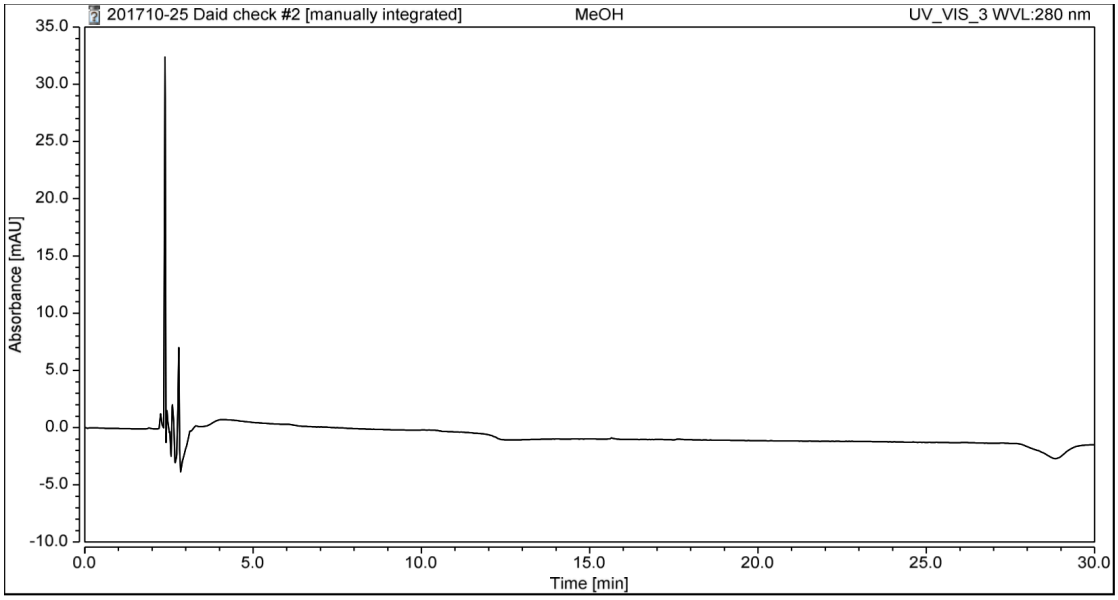


Figure 3.4: A typical HPLC trace of the sample blank, HPLC grade MeOH. Detection at λ 280 nm.

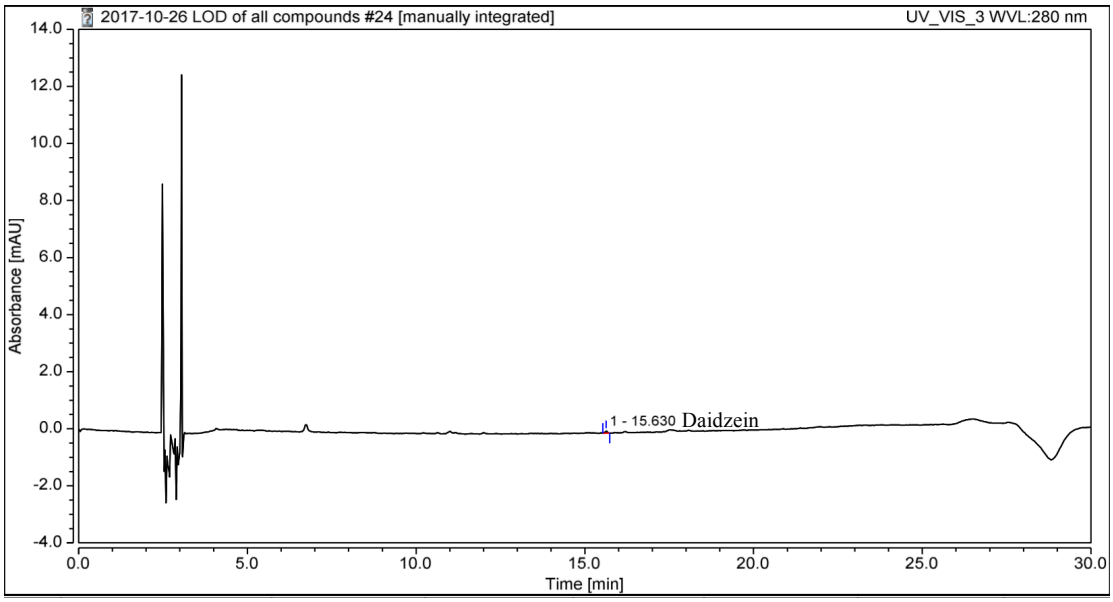


Figure 3.5: Typical HPLC trace showing the LOD (0.0025 mg/L) for daidzen (t_R = 15.630) in HPLC grade MeOH. Detection at λ 280 nm.

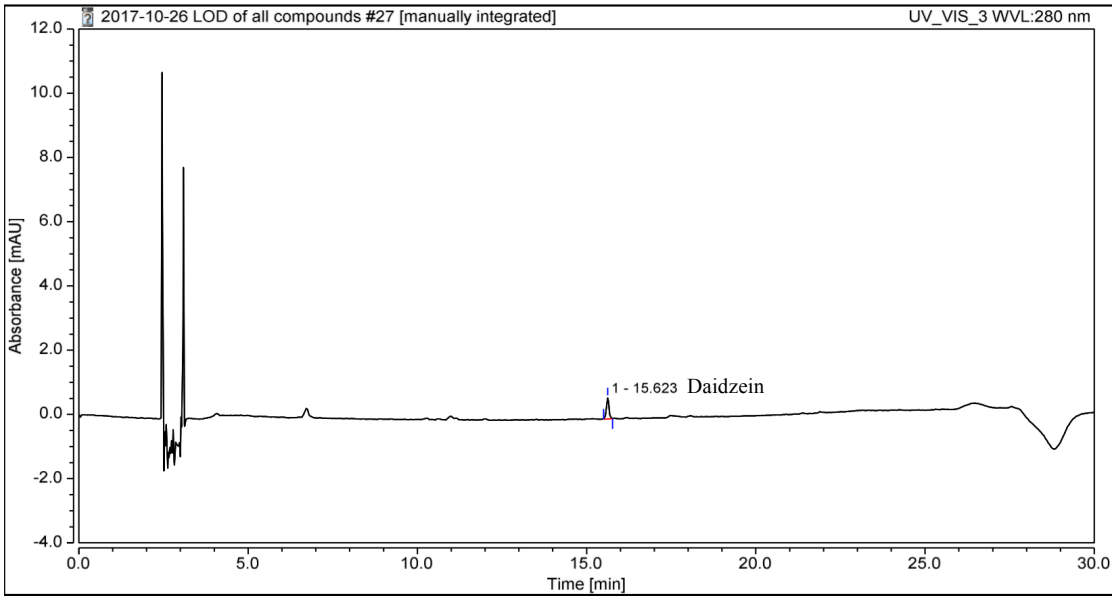


Figure 3.6: Typical HPLC trace showing the LOQ for dadzein (t_R = 15.623 min) (0.05 mg/L) in HPLC grade MeOH. Detection at λ 280 nm.

3.5 Calibration graphs for Endogenous Estrogens and Xenoestrogens

The calibration curves for E2, daidzein, formononetin, zearalanone, genistein, EE2, BPA and equol all have good linear regression with R^2 values in the range of 0.97 – 0.99 (Fig. 3.7). The linear nature of the calibration graphs allows for the reliable calculation of concentrations of E2, daidzein, formononetin, zearalanone, genistein, EE2, BPA and equol concentrations in the range of 0.05 – 1 mg/L.

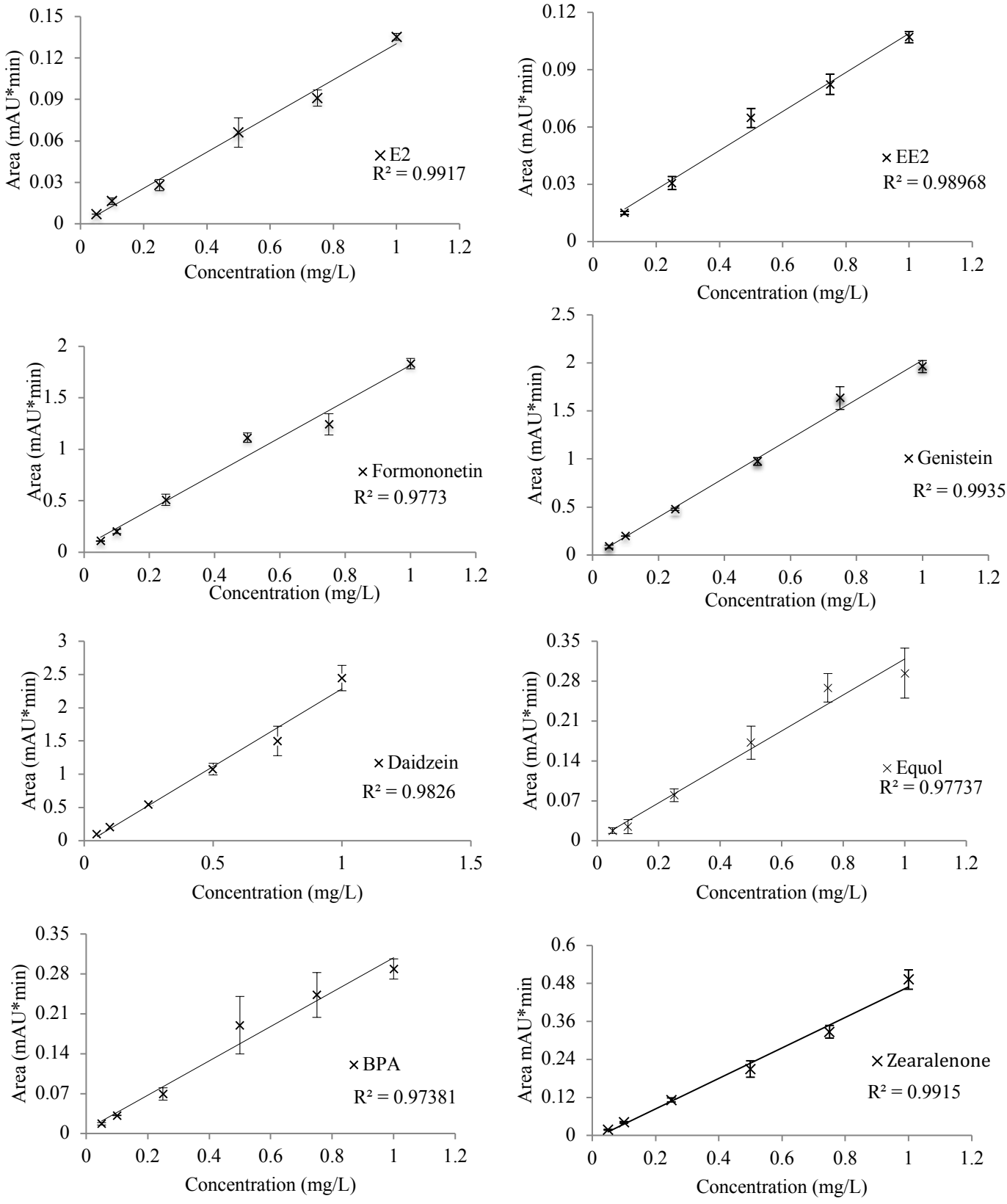


Figure 3.7: Calibration graphs for E2, daidzein, formononetin, zearalanone, genistein, EE2, BPA, and equol authentic standards. All show good linear regression with R^2 values in the range of 0.97 – 0.99 (n = 3).

3.6 Extraction Recoveries

Milk that was not found to contain xenoestrogens in previous analyses (i.e. xenoestrogen-free) was used for the recovery experiments. The recoveries for E2, EE2, formononetin, daidzein genistain, equol, BPA, and zearalenone were carried out by spiking milk samples found to not contain xenoestrogens and extracted follow the method outlined in Section 2.3.1, Method 1. Spiked milk sample extracts were analysed using HPLC-DAD (see Section 2.5). The extraction recoveries are presented in Table 3.5. Recoveries range from 62 % for BPA to 94% for EE2.

Table 3.5: Mean recoveries of xenoestrogens spiked at 1 mg/L in xenoestrogen-free milk (n = 3).

Compound	**Mean Recovery ± SD (%)**
E2	67.44 ± 8.0
EE2	94.29 ± 12.38
Formononetin	83.70 ± 9.56
Daidzein	72.46 ± 13.69
Genistein	91.13 ± 9.19
Equol	78.84 ± 9.56
BPA	61.96 ± 7.81
Zearalenone	87.74 ± 12.52

3.7 Analysis of Endogenous Estrogens and Xenoestrogens in Milk

E2, EE2, formononetin, daidzein, genistein, equol, BPA, and zearalenone were measured in extracted milk samples using HPLC-DAD (see Section 2.5), a typical chromatogram of an extracted milk sample is shown in Fig 3.8. A total of 30 bottles of milk purchased at a local supermarket were extracted and analysed (Table 3.6).

Table 3.6: Calculated peak areas and concentrations of endogenous estrogens and xenoestrogens (E2, EE2, formononetin, daidzein, genistein equol, BPA, zearalenone) in 30 commercially available New Zealand Cow's milk samples measured using HPLC-DAD.

Sample	Peak Area (mAU)/ Concentration in mg/L							
	E2	**EE2**	**Formononetin**	**Daidzein**	**Genistein**	**Equol**	**BPA**	**Zearalenone**
1	ND	ND	ND	ND	ND	ND	ND	ND
2	ND	ND	ND	ND	ND	ND	ND	ND
3	ND	ND	ND	ND	ND	ND	ND	ND
4	ND	ND	ND	ND	ND	ND	ND	ND
5	ND	ND	ND	ND	ND	ND	ND	ND
6	ND	ND	ND	ND	ND	ND	ND	ND
7	ND	ND	ND	ND	ND	ND	ND	ND
8	ND	ND	ND	ND	ND	ND	0.659/ 0.041	ND
9	ND	ND	ND	ND	ND	ND	ND	ND
10	ND	ND	ND	ND	ND	ND	ND	ND
11	ND	ND	ND	ND	ND	ND	ND	ND
12	ND	ND	ND	ND	ND	ND	ND	ND
13	ND	ND	ND	ND	ND	ND	ND	ND
14	ND	ND	ND	ND	ND	ND	0.625/ 0.039	ND

15	ND	ND	ND	ND	ND	ND	0.293/ 0.019	ND
16	ND	ND	ND	ND	ND	ND	0.146/ 0.010	ND
17	ND	ND	ND	ND	ND	ND	0.200/ 0.013	ND
18	ND	ND	ND	0.185/ 0.021	ND	ND	Detected	ND
19	ND	ND	ND	ND	ND	ND	0.343/ 0.022	ND
20	ND	ND	ND	ND	ND	ND	0.110/ 0.007	ND
21	ND	ND	ND	ND	ND	ND	ND	ND
22	ND	ND	ND	ND	ND	ND	ND	ND
23	ND	ND	ND	ND	ND	ND	ND	ND
24	ND	ND	ND	ND	ND	ND	ND	ND
25	ND	ND	ND	ND	ND	ND	ND	ND
26	ND	ND	ND	ND	ND	ND	0.112/ 0.008	ND
27	ND	ND	ND	ND	ND	ND	ND	ND
28	ND	ND	ND	ND	ND	ND	ND	ND
29	ND	ND	ND	ND	ND	ND	ND	ND
30	ND	ND	ND	ND	ND	ND	ND	ND

ND. = Not detected

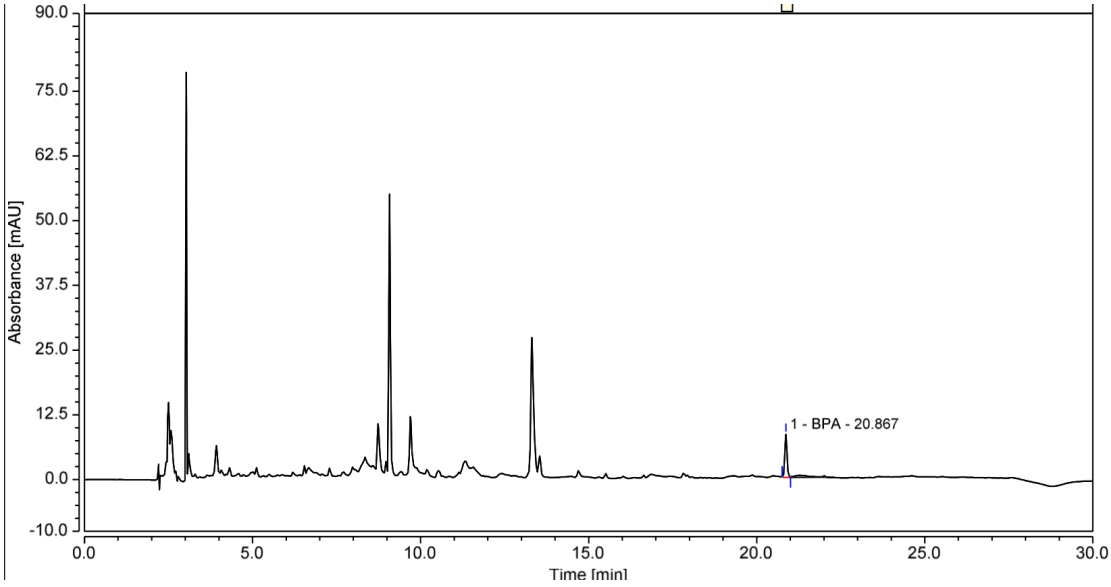


Figure 3.8: A typical HPLC trace of an extracted milk sample showing a BPA peak at t_R = 20.867 min using HPLC-DAD. Detection at λ 280 nm.

3.7.1 Source of BPA in Milk Sample Extracts

The source of BPA was investigated by contacting a milk company and a farming equipment. Meadow Fresh were contacted about what their plastic milk bottles and cardboard milk cartons were made of. An employee of Meadow Fresh stated that the plastic milk bottles are made of food grade high-density polyethylene (HDPE), and the milk cartons are made of paper with a poly ethylene liner.

Skellerup were contacted regarding farming equipment, in particular the claw bowl and milk tubing. Skellerup were contacted by email on 3 occasions, email replies were received indicating that my enquiry had been passed on the technical manager and general manager, and to expect a reply. However, no reply was received.

3.8 Metabolite identification

An unidentified peak often appeared in milk sample extracts (t_R = 13.5 min) (Fig. 3.9). It was thought that the unidentified peak might be a metabolite of equol because the UV spectrum obtained by HPLC-UV was similar to the UV spectrum of the authentic

standard of equol. The UV maximum of equol was 196, 221, and 281, compared with the unknown peak of 193, 228, and 272.

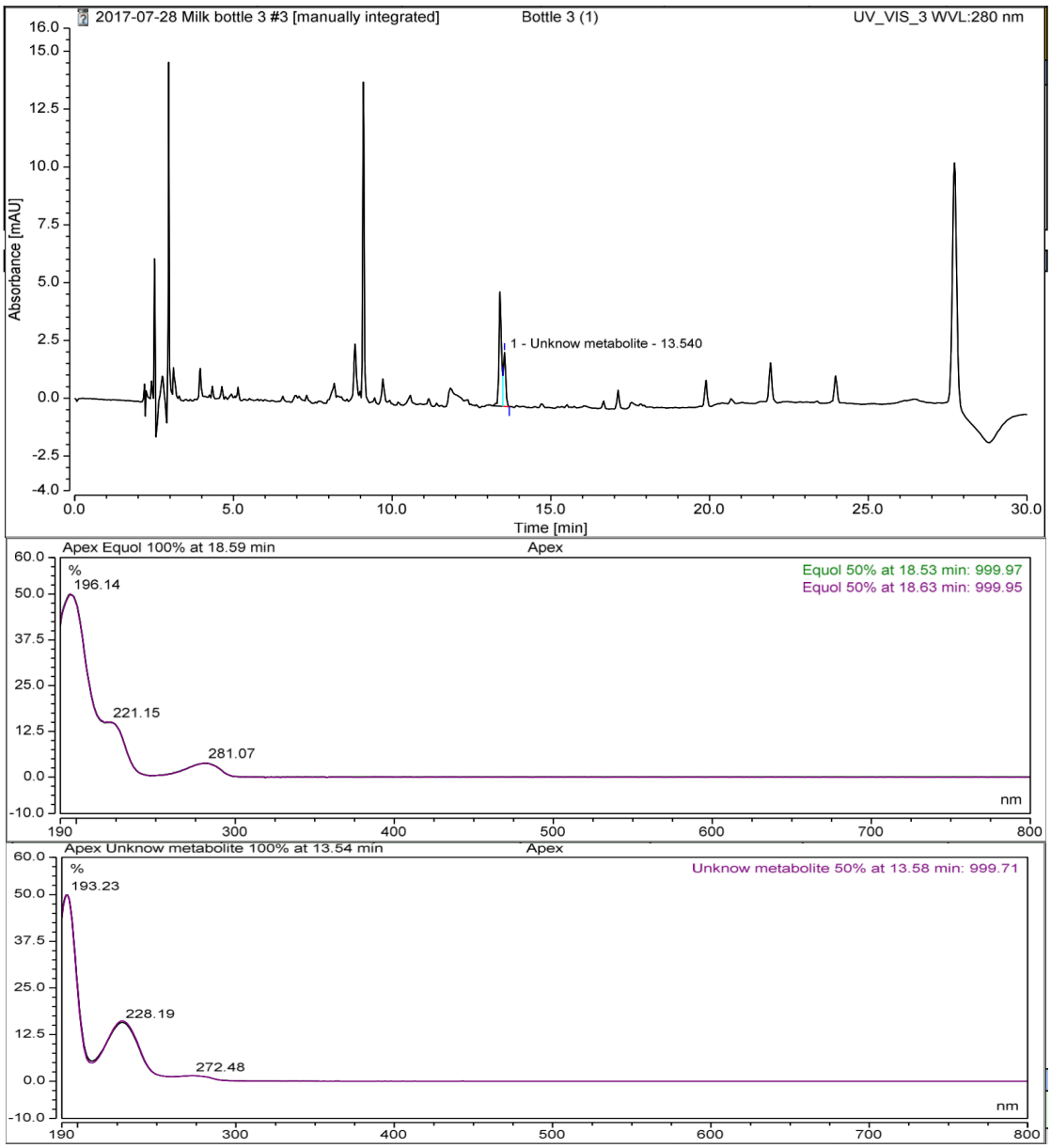


Figure 3.9: Typical HPLC trace of a milk sample extract showing that unidentified peak, suspected to be a metabolite (top) with a t_R = 13.540 min. The UV spectrum of the unknown peak (bottom) is similar to equol (top), sharing similar UV maxima, but slightly shifted.

3.8.1 Identification of the suspected metabolite

Mass Spectrometry

The suspected metabolite peak was collected using the fraction collection function on the HPLC system. The fraction was analysed by direct inject probe mass spectrometry using a Maxis 4G spectrometer operated in high resolution mode with a positive ESI source. Analysis was carried out by Dr. Marie Squire. The fraction collected peak (5 μL) had an ion of 349.1263 m/z (Fig. 3.10).

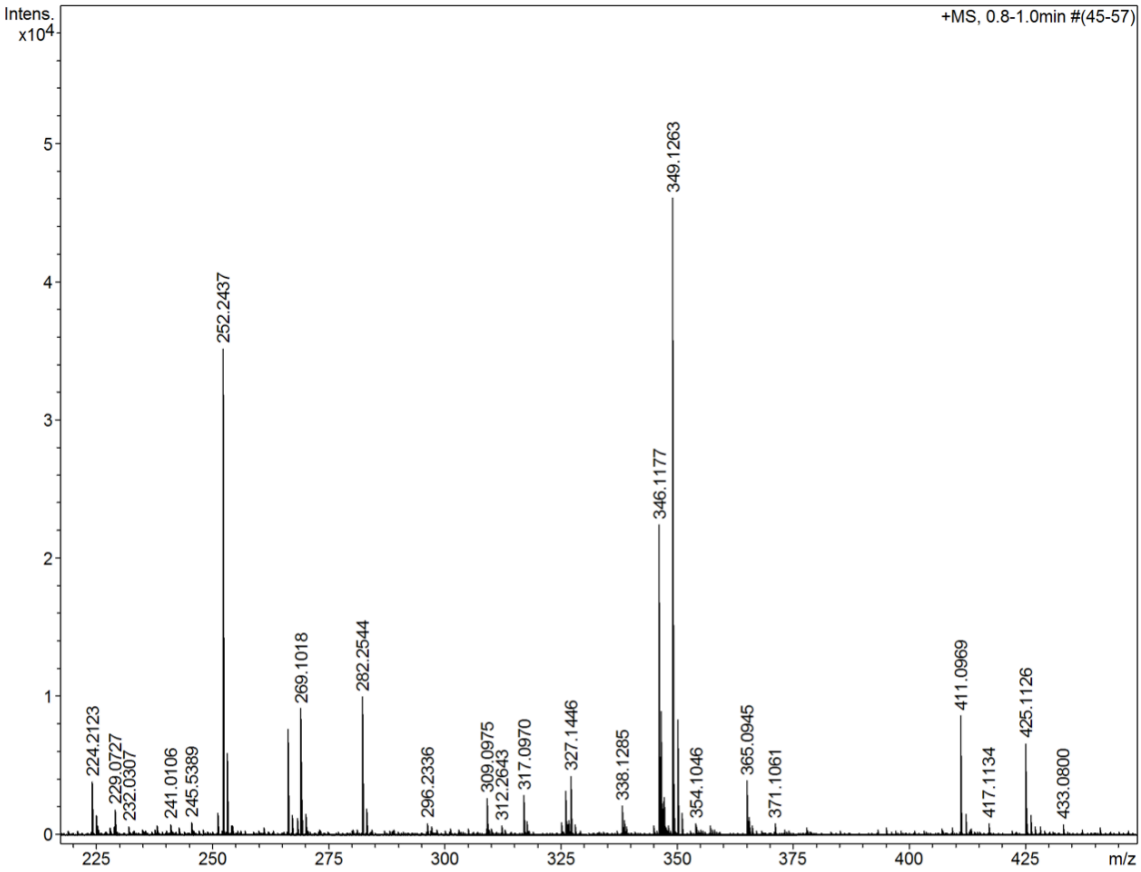


Figure 3.10: Mass spectrum of the unidentified peak; a suspected metabolite of equol, with m/z = 349.1263

3.8.2 Nuclear Magnetic Resonance Spectroscopy

The suspected metabolite peak fraction was dried at 60°C with a stream of nitrogen and the residue dissolved in 2 mL [U-^{2}H]-DMSO and subjected to nuclear magnetic resonance spectroscopy (NMR). Unfortunately, the concentration of the compound was too low, and only solvent peaks were seen (Fig. 3.11).

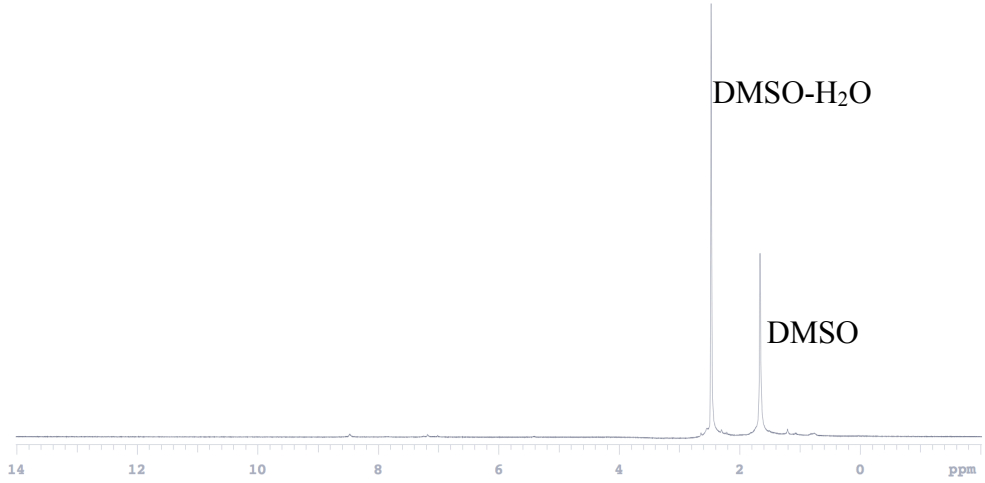


Figure 3.11: ^{1}H NMR spectrum of the suspected metabolite peak fraction in 2 mL [U-^{2}H]-DMSO. Only the solvent peaks, DMSO and DMSO-H_2O were observed due to the concentration of the suspected metabolite being too low.

3.9 Release of Xenoestrogens by De-Conjugation and Enzyme Digest

3.9.1 Cleavage of Sulfated and Glucuronidated Xenoestrogens using the Enzyme Sulfatase/β-Glucuronidase from *H. pomatia*

Milk purchased from a local supermarket was incubated with sulfatase/β-glucuronidase overnight buffered at pH 5.0 at 37°C in a shaking incubator extracted and analysed by HPLC-DAD. A peak of genistein (t_R = 18.56 min) appeared in extracts of milk treated with sulfatase/β-glucuronidase. Genistein was at a concentration of 0.062 mg/L. A

typical chromatogram of milk extracted after incubation with sulfatase/β-glucuronidase is shown in Figure 3.12.

3.9.2 Release of Xenoestrogens by Proteinase K Digestion

Milk purchased from a local supermarket was incubated with proteinase K overnight buffered at pH 8.0 at 60°C in a shaking incubator extracted and analysed by HPLC-DAD. No extra identifiable peaks were present in extracts of milk treated with proteinase K. A typical chromatogram of milk extracted after incubation with proteinase K is shown in Figure 3.12.

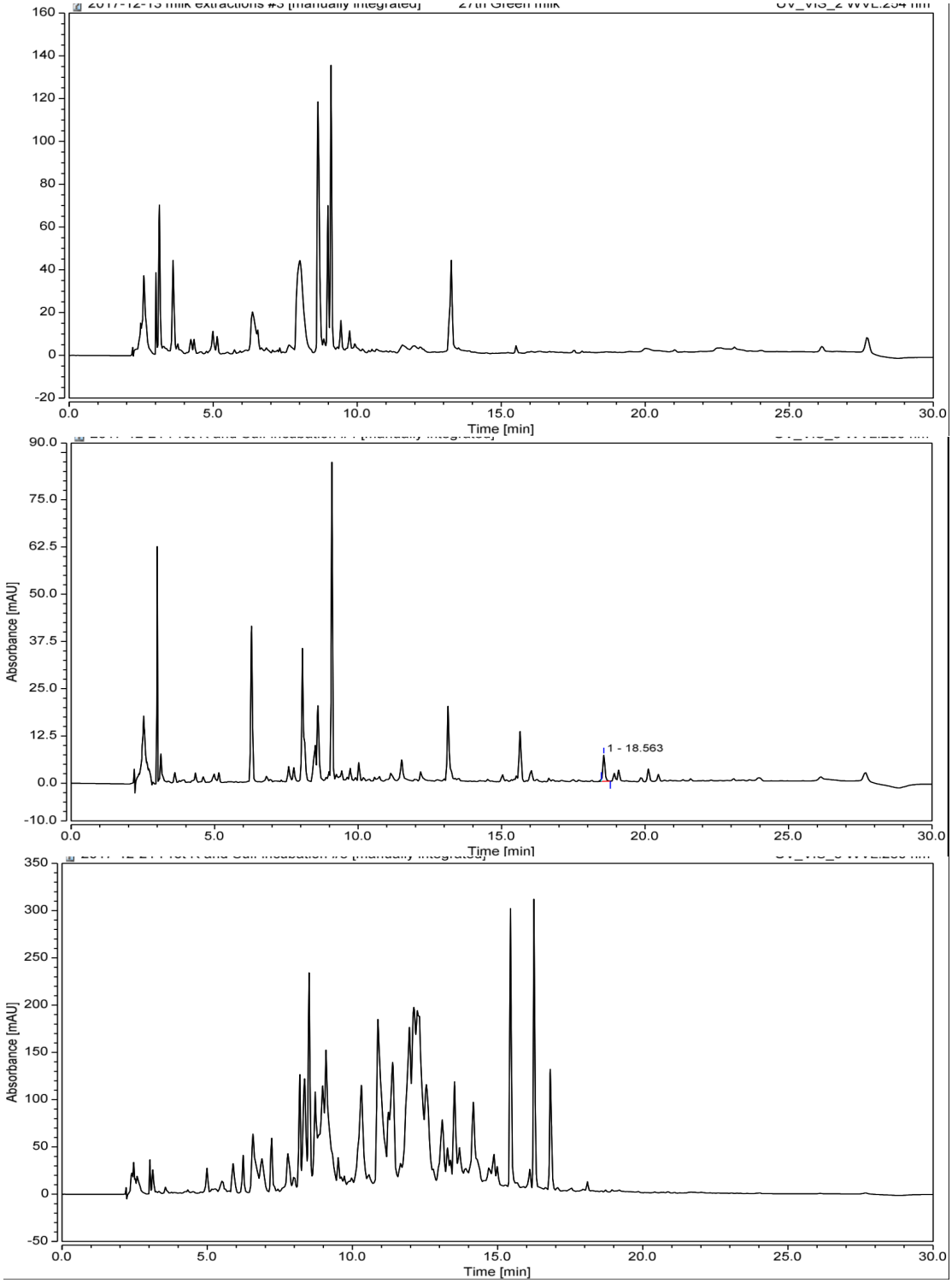


Figure 3.12: A typical HPLC chromatogram of before (top) and after extracts of milk treated with sulfatase/β-glucuronidase (middle), and proteinase K (bottom). A peak of genistein (t_R = 18.56 min) appeared after treatment with sulfatase/β-glucuronidase (top). No extra identifiable peaks appeared after treatment with proteinase K (bottom).

3.10 Extraction and Analysis of Glyphosate in New Zealand Milk

Glyphosate residues were measured in extracted milk samples purchased from a local supermarket and extracted following the method outlined in Section 2.5.1. Analysis of extracted milk samples was carried out by LC-MS (Section 2.6.1). LC-MS of glyphosate standards did not produce an ion consistent with glyphosate; therefore, the unusual step of direct inject probe mass spectrometry because an ion consistent with glyphosate produced (0.001 mg/L, Fig. 3.13). An extracted milk sample was analysed by direct inject probe mass spectrometry; no ion was produced consistent with the ion produced in the glyphosate standard (Fig. 3.14).

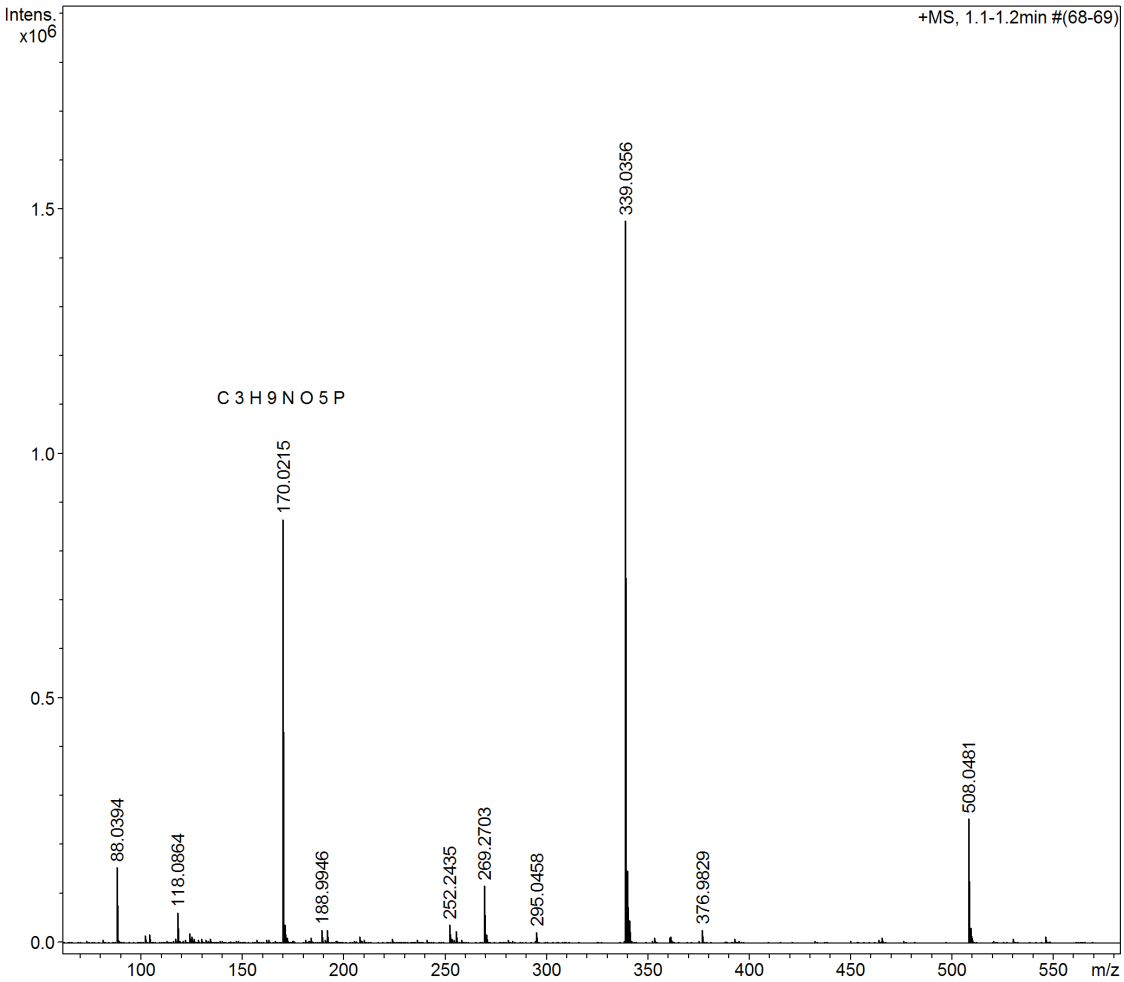


Figure 3.13: Mass spectrum of a glyphosate standard (0.001 mg/L) in Milli-q water by direct inject probe mass spectrometry. Glyphosate m/z = 170.0215.

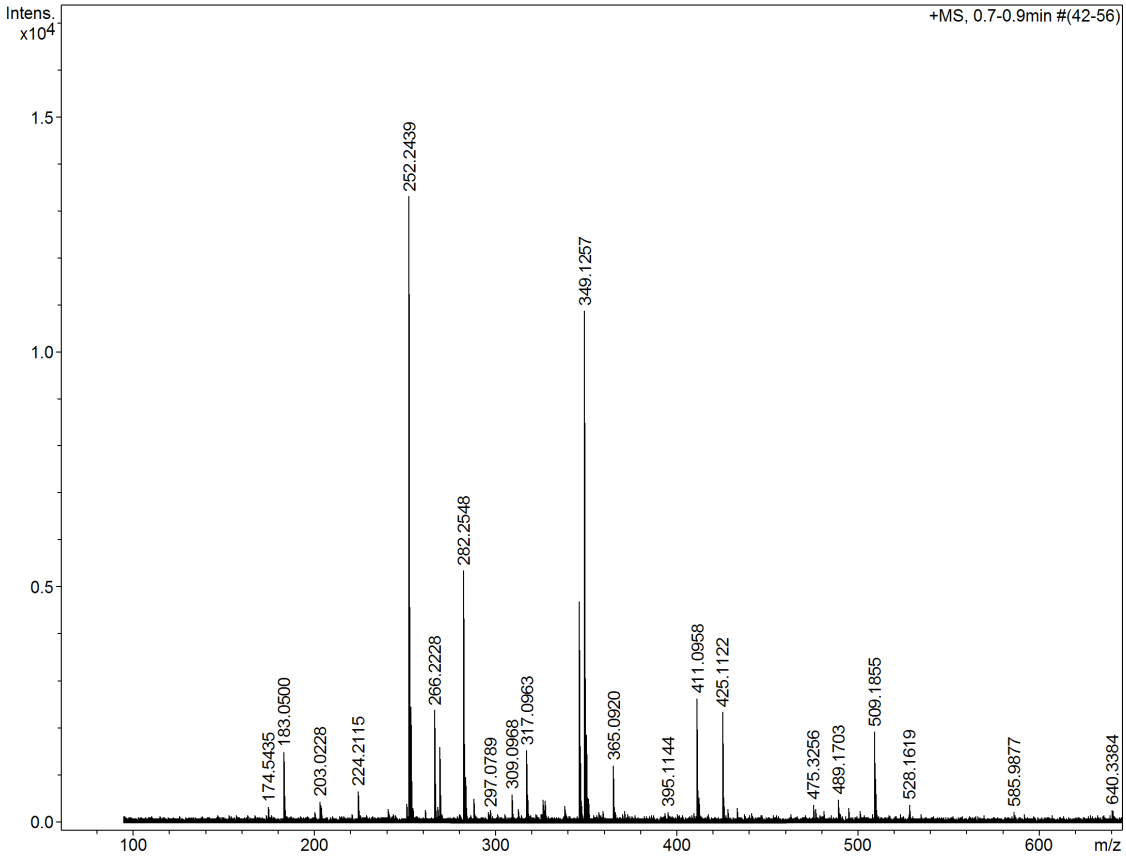


Figure 3.14: A typical mass spectrum of a milk sample extract analysed by direct inject probe mass spectrometry. No ions were produced which matched the ion produced in a glyphosate standard.

3.11 Metabolism of Xenoestrogens with Cow's Rumen Model System

Wilkins Chalgren broth spiked with formononetin (10 mg/L) was incubated with cow rumen slices (4 x 3 cm) sampled daily for 3 days. The incubate was extracted and analysed by HPLC-DAD. A suspected equol peak appeared after the incubation with cow rumen slices not identified in controls (Fig. 3.15). The identity of equol was confirmed by spiking extracts with equol authentic standard. This suggests that the model is able to generate equol, a known microbiome metabolite of formononetin.

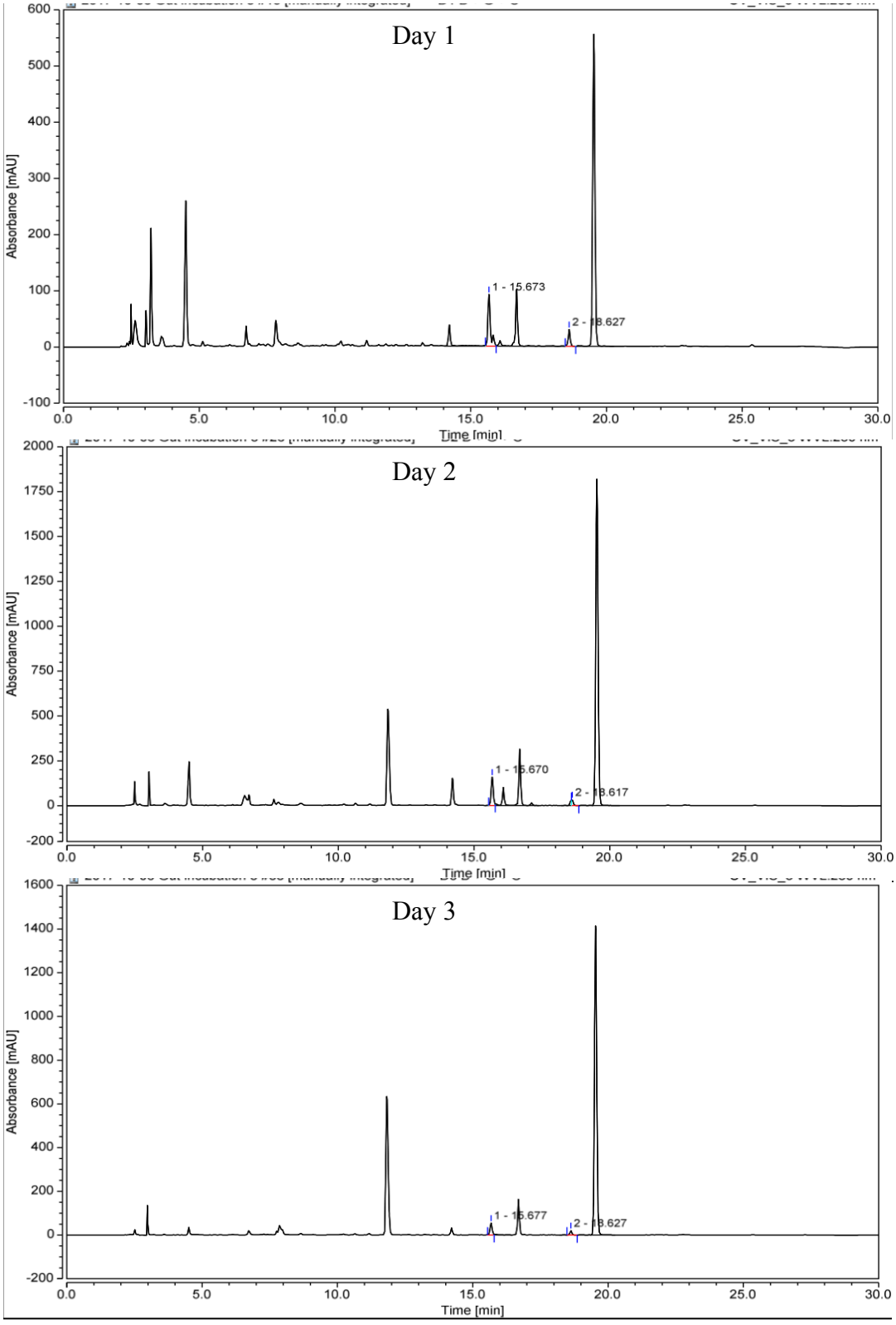


Figure 3.15: Typical HPLC trace of day 1,2, and 3 sample extracts of cow rumen slices (4 x 2 cm) incubated with formononetin (t_R = 15.6 min). A suspected equol peak (t_R = 18.6 min) was produced and confirmed by spiking with equol authentic standard.

3.12 *In-silico* Glyphosate/ERα Binding Studies

Using ERα X-ray crystal structure coordinates (1ERE) a model of human ERα was built in Schrödinger (see Section 2.8). This was used for glyphosate docking studies. Rigid docking was used. This means that the conformation of the receptor is the same as the ERα/E2 complex and is used to determine the fit of another potential ligand to the LBC arranged to bind E2. This results in less favourable calculated binding energies or no pose docked. In contrast, more flexible ligands have more poses within the LBC, the given calculated binding energies show the conformation of ligand that gives more favoured interactions between the ligand and receptor complex.

The *in-silico* model shows that glyphosate has some structural analogy with E2 in the ERα active site (Fig. 3.14), with a docking score of -21.55 kJ/mol compared to E2 which has a docking score of -41.76 kJ/mol. Glyphosate (Fig. 3.16) orientates in the LBC in such a way that the two -OH groups of the phosphate group hydrogen bonds with Glu353, and one phosphate -OH group hydrogen bonds with Arg394 (Fig. 3.16). The -NH- group of glyphosate forms a hydrogen bond with Leu346, which is not seen when E2 is bound (Fig. 3.14). One other key difference is that glyphosate does not interact with His524, whereas E2 does (Fig. 3.16). The distance of glyphosate from His524 is 8.67 Å and 9.90Å, this is likely too distant to facilitate hydrogen bond formation.

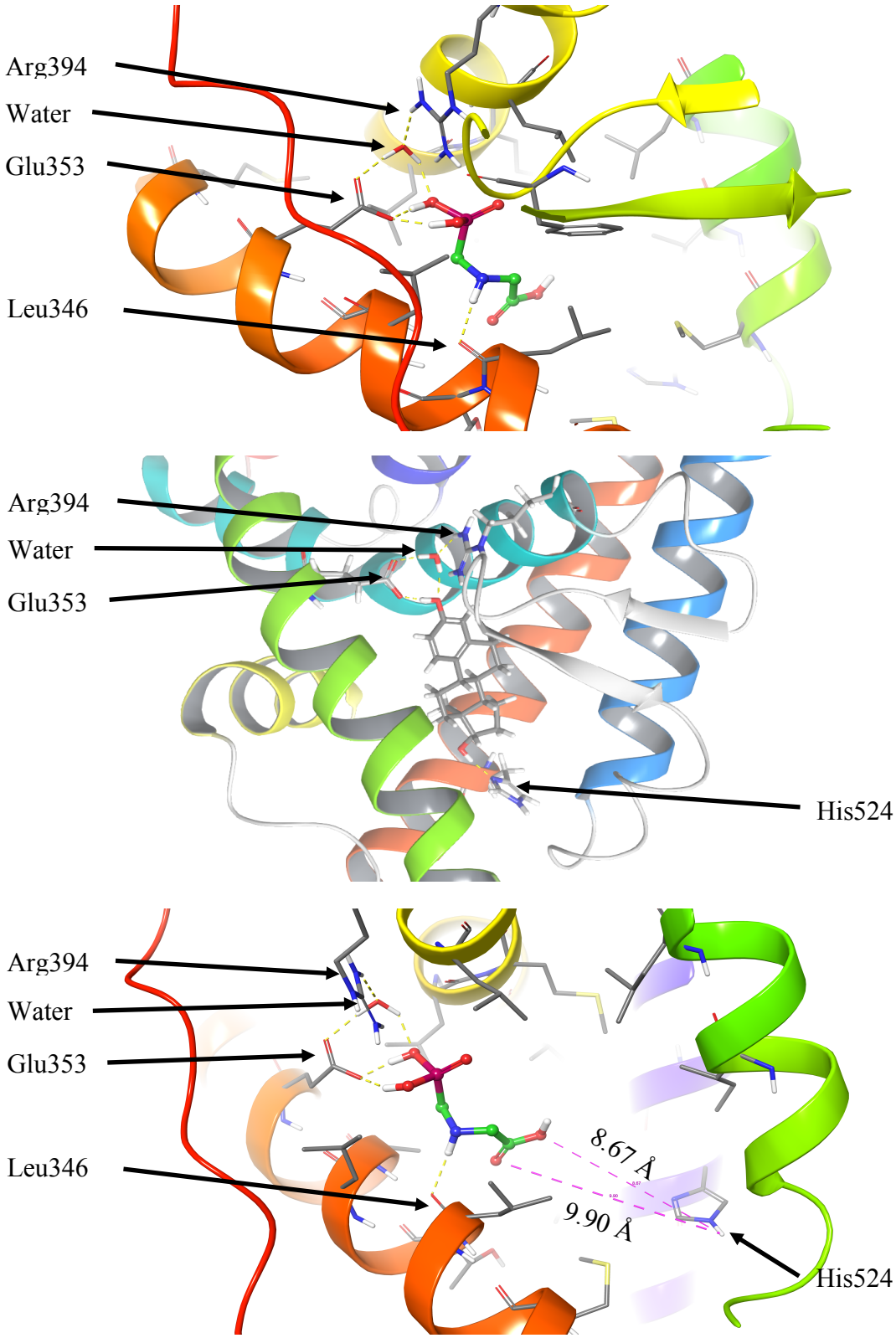


Figure 3.16: Glyphosate (top) bound in the LBC of ER shows similar bonds as E2 (middle) bound (Glu353, Arg394, and a water molecule), with an extra bond to Leu346 that does not form with E2. Glyphosate (top and bottom) does not form a hydrogen bond to His524 which E2 (middle) does, and is far away from the residue in the LBC (8.67 Å and 9.90 Å)

Chapter 4 – Discussion

Chapter 4 – Discussion

The aim of this study was to carry out a risk assessment of New Zealand cow's milk in an estrogen mimic context. The study successfully evaluated methods for the extraction of endogenous estrogens and xenoestrogens. The chosen method was used for the extraction and measurement of endogenous estrogens (E2) and xenoestrogens (EE2, formononetin, genistein, daidzein, equol, BPA) in New Zealand cow's milk samples. The method had recoveries ranging from 62 – 94%, LODs ranging from 0.0025 – 0.1 mg/L and LOQs ranging from 0.05 – 1 mg/L for the target compounds. The chosen method identified genistein, daidzein, and BPA in New Zealand milk sample extracts at concentrations ranging 0.007 – 0.041 mg/L. In milk sample extracts a peak of an unknown identity often appeared. This peak had a t_R = 13.540 min and a UV spectrum very similar to equol; however, it eluted sooner than equol. It was thought that this peak might be a equol metabolite. The unidentified peak was isolated by HPLC fraction collection and investigated by MS and NMR. The mass spectrum of the residue was 349.1263 m/z; however, no information could be gained from NMR due to the low concentration of the residue. The unidentified peaks identity was speculated based on common metabolism route and m/z; however, was not identified. The presence of glyphosate in cow's milk was investigated in light of the presence of glyphosate in ice cream samples for the United Kingdom. A previously accepted extraction method was used to determine the presence of glyphosate, due to the low concentrations published, direct inject probe mass spectroscopy was utilised. In New Zealand cow's milk samples (n = 5), no mass ion consistent with glyphosate was identified. A cow gut model system was set up and used to investigate the metabolism of phytoestrogens. Briefly. Cow rumen slices were cut into strips (4 x 2 cm) and incubated with anaerobic broth spiked with phytoestrogens (10 mg/L). A phytoestrogen found in clover, formononetin, was incubated in this system. When the broth containing formononetin was extracted, a well-known gut microbiota metabolite, equol was identified. This indicates that the system is capable of producing metabolites during incubation However, no unknown metabolites of formononetin were identified.

4.1 Evaluation of Extraction Methods for the Extraction of Xenoestrogens from Cow's Milk

It is well documented in scientific literature that endogenous estrogens and xenoestrogens are present in cow's milk; therefore, there has been methods developed previously for the extraction of these compounds in cow's milk.[12-15, 17, 20-22, 133, 152, 155, 157, 158, 210, 212-218, 220-236] In view of this, it was decided that one of the available methods would be used and modified for this study. This was decided because developing a new method can be laborious and time consuming. The purpose of the pilot study was to investigate the presence of endogenous estrogens and xenoestrogens in milk, not develop a new method to do this. The study modified published methods by using HPLC-DAD rather than LC-MS because of the ease of data analysis by HPLC-DAD. LC-MS is a highly sensitive analytical technique; it produces a peak for any component in the sample which is volatile. Manual peak analysis is therefore required. The MS can also cause the compound to split leading to m/z which might not be expected. In extracted milk samples where there is more than one compound, this could lead to the compounds being missed. Whereas, a compound requires a chromophore for HPLC-DAD to be detect. HPLC-DAD can further discriminate based on the UV maximum of compounds by setting the detection wavelength. This allows for more discrimination of results as all compounds of interest could be analysed at one wavelength. Therefore, data analysis is cleaner, easier, and quicker by HPLC-DAD due to it being more selective on the data displayed.

Methods for the extraction of endogenous estrogens and xenoestrogens were chosen based on their attributes (e.g. time taken to carry out, robustness). Many of the methods published in literature are designed to extract a single group of compounds); for example, Goyon *et al.* published a method for the determination of steroid hormones (e.g. E1, E2, E3, progesterone, testosterone) and Steinshamn *et al*. published a method for the determination phytoestrogens (e.g. formononetin, equol, genistein).[155, 235] Methods which are designed to extract one group of compounds should have better recoveries compared with a method which extracts all groups of xenoestrogens. The reason for this is because each group of compounds have different properties (e.g. solubility); therefore, different solvents are used to achieve the maximum extraction of

compounds. This is important when extracting endogenous estrogens and xenoestrogens from milk. Milk is a high fat matrix which these compounds are very soluble in due to their hydrophobic nature; therefore, choosing a solvent which the target compounds are more soluble in compared with the lipid in milk needs to be selected. However, not all groups of compounds will be equally soluble in the chosen solvent, which leads to different recovery efficiencies. Thus, it is likely that multiple methods should be used to achieve the best recoveries for all groups of compounds; however, this is not cost or time efficient, unless only one group of compounds are wanting to be measured. The aim of the study was to evaluate the risk of New Zealand cow's milk in an estrogen mimic context; therefore, methods capable of extracting a broad range of endogenous estrogens and xenoestrogens were investigated.

4.1.1 Evaluation of Methods for the Extraction of Endogenous Estrogens and Xenoestrogens from Cow's Milk

Three extraction methods were evaluated for the extraction of endogenous estrogens and xenoestrogens from cow's milk. This allowed for the best method capable of extracting the study's target compounds to be picked. Of the three methods chosen to investigate, two of these were designed to extract a broad range of compounds. These were Method 1 published by Socas-Rodríguez *et al.*, 2017, and Method 3 published by Wielogorska *et al.*, 2014.[207, 208] The other method selected was one which extracted only endogenous estrogens; this was published by Malekinejad *et al.*, 2006 (Method 2).[12] One targeted extraction method for endogenous estrogens was picked to compare differences in effectiveness of a targeted extraction method compared with a broad compound extraction method. A brief description of these are shown in Table 4.1. After these extraction methods were investigated, Method 1 was chosen to be used to extract endogenous estrogens and xenoestrogens form cow's milk samples. The reason for choosing this method was because it extracted a broad range of compounds and was quick to carry out compared with the other investigated methods.

Table 4.1: Summary of the three chosen methods for the extraction of endogenous estrogens and xenoestrogens from New Zealand cow's milk samples.

Method 1 (Socas-Rodríguez *et al.*, 2017)	**Method 2** (Malekinejad *et al*., 2006)	**Method 3** (Wielogorska *et al.*, 2014)
10 mL Milk + 15 mL ACN, shaken, + 6 g $MgSO_4$ + 1.5 g NaCl, shaken, centrifuge, take supernatant + 500 mg C18 sobent + 1.8 g $MgSO_4$, shaken, centrifuge, evaporate, dissolve in 50 % MeOH, analyse.	10 mL milk + 80 % (v/v, aq) MeOH, vortex, centrifuge, bottom layer kept + hexane centrifuge, top layer kept, + DCM to remaining liquid, vortex, slow shaking, centrifuge, lower layer kept, repeat DCM step, combine lower layers, vortex, evaporate with stream of N_2 (g), dissolve in MeOH, vortex, + Milli-Q, C18 SPE column, dry SPE column (stream of N_2 (g)), elute with MeOH, evaporate, dissolve in 50% MeOH, analyse.	10 mL milk + 1 % (v/v, aq) acetic acid in ACN + 2.5 g $MgSO_4$ + 1.0 g NaCl, shaken, centrifuge, supernatant evaporate with stream of N_2 (g), dissolved in Milli-Q, C18 SPE column, column washed, analytes eluted with 1% (v/v, aq) acetic acid in 10% (v/v) MeOH in TBME, evaporate with stream of N_2 (g), dissolve, in 50 % MeOH, analyse

Reasons for Selecting Method 1

All methods had 3 major components to them: 1) defatting of the milk; 2) extraction and clean-up of the target compounds; 3) concentrating and analysis of compounds. The slowest of the three selected methods was Method 2, with the fastest being Method 1. Milk samples extracted using Method 2 used two rounds of LLE and had the most steps in the extraction, which is likely why it was the longest to carry out. Method 1 was likely the fastest to carry out because a rotary evaporator was used to dry solvents, and the SPE clean-up was carried out by using a centrifuge. Whereas, Methods 2 and 3 used a stream of N_2 gas to dry solvents, and a gravitational column to carry out SPE.

The cost to carry out these 3 methods was not calculated, but, it would be assumed that Method 3 would be the most expensive to carry out because it uses the most chemicals. However, the cost of a method should not impact a decision on what method to use if it produces better results.

The most important consideration of a methods is the results which the method produces. It is important that the method has repeatable accuracy, precision and robustness; if not, then the method is not useable. In preliminary milk sample extracts, Method 1 HPLC-DAD chromatograms were more repeatable compared with the other methods. HPLC chromatograms of milk sample extracts using Method 1 also had a lower sound to noise ratio after t_R = 13 min. This is important because endogenous estrogens and xenoestrogens are eluted after 13 min using the study's HPLC method. If there was high noise at the same time as a compound eluted, this can lead to an over estimation of a compounds concentration due to a broad peak, or the compound could be completely missed; therefore, leading to discrepancies.

Method 1 was chosen for the study to carry out the extraction of endogenous estrogens and xenoestrogens from New Zealand cow's milk because it can extract these compounds, it is quick and easy to carry out, and the results are repeatable with low noise in HPLC chromatograms where the compounds are known to elute.

4.1.2 Validation of Selected Method

For the extraction of endogenous estrogens and xenoestrogens, Method 1 was selected because of its ability to extract a broad range of estrogens and estrogen mimics, speed of extraction, and reliability.

The first step of Method 1 was to remove any unwanted proteins in the milk, minimising the matrix effects. If the protein is not removed from milk it can lead to contamination, blockages and damage/adsorption to the stationary phases of chromatography columns. In Method 1, precipitation of the protein was achieved by the addition of acetonitrile, followed by centrifugation. This caused three layers to appear with the proteins forming

a solid precipitate layer in the centrifuge tube. The layer containing the residues of interest was the top layer; this was transferred to a new centrifuge tube for the clean-up step. Recently, a novel method was developed for the extraction of estrogens from milk samples using a nanocomposite of polypyrrole-coated nanoparticles (a nanoscale particle), removing the requirement for deproteination.[216] This is because the polypyrrole coating has a highly π-conjugated structure and is hydrophobic. This attracts and captures the endogenous estrogens from milk samples. The method had very low limits of detection (5.1×10^{-6} – 6.6×10^{-5} mg/L) with LC-MS and high recoveries (83.4% – 108.5%) of endogenous estrogens.[216] However to make these particles, they require synthetic chemistry. A solvothermal process is used, in which the precursors are mixed in a Teflon-lined stainless steel autoclave which is heated and then allowed to cool.[216] If these particles could be adapted to extract other xenoestrogens, it could be a very useful method to use because it is quick to carry out, and produced low detection limits and good recoveries.

Following deproteination, the analytes require further cleaning up by removing any remaining peptides, trace metals and other milk components which may still be present in samples. This is achieved either by liquid-liquid extraction (LLE) or SPE. In all of the selected methods, SPE was used. SPE is preferred over LLE because it is quicker, cheaper and easier to carry out. The reason that LLE is slower and more difficult to carry out is because of the larger amount of solvent required compared to SPE. The less solvent required for SPE leads to higher recoveries due to more of the matrix interferences retaining on the stationary phase instead of overloading the column and passing through.[208, 218, 237] SPE is quicker due to smaller amounts of solvent is required; therefore, drying down extracts is faster. The most common stationary phase used for SPE is Oasis HLB sorbents. These have become the standard for methods developed for the extraction of endogenous estrogens and xenoestrogens from food matrices (e.g. milk, breast milk, honey, cheese) because of their effectiveness.[14, 207, 208, 215, 222, 238, 239]

An important feature of the selected extraction method was the reproducibility of low noise in HPLC chromatographs. These had low noise between the t_R of 13 – 29 min. It was important that this time period was clear because endogenous estrogens and xenoestrogens had retention times between 15 min – 28 min. The step of the method

responsible for this low sound to noise ratio in chromatographs was the clean-up component. This removed any matrix interferences that were left after the deproteination step. Removing the matrix interferences means the background noise is kept low allowing the low levels of endogenous estrogens and xenoestrogens to be detected and measured accurately.

The evaluation of Method 1 indicates that it was the best choice for the extraction of endogenous estrogens and xenoestrogens from cow's milk samples. Importantly, this method extracted xenoestrogens from cow's milk in the study. As the name of the method suggests, it is quick, easy, cheap, effective, rugged, and safe. This was the quickest method to carry out with no difficult steps to carry out. The method was effective and rugged because the HPLC chromatograph consistently had low noise, and successfully extracted endogenous estrogens and xenoestrogens from spiked milk samples. This method was successful in producing low noise HPLC chromatograms in both full fat and skim milk showing that it is not limited to low fat samples. All compounds have clear separation with sharp peaks with HPLC-DAD analysis.

Recovery of Target Compounds

An indication of how well an extraction method performs is by recovery of authentic standards from spiked milk samples. The recoveries for this study are shown in in Section 3.6 (Table 3.5). The study produced a large range of recoveries for the target compounds; BPA had the lowest recovery of 61. 96 ± 7.81%, whereas the recovery of EE2 was 94.29 ± 12.38%. Overall the recoveries were split into two groups: compounds with recoveries not close to 100% and compounds close to 100%. The compounds that had recoveries not close to 100% were E2 (67%) daidzein (72%) and BPA (62 %). The compounds that had recoveries close to 100% were EE2 (94%), formononetin (84%), genistein (91%), equol (79%), and zearalenone (88%). Even though this method produced mixed recoveries for the target compounds, they are in line with published literature using methods which have been accepted for milk sample analysis.[12, 158, 213, 214, 217, 222] One exception in the stud was BPA; these study's report recoveries 20% higher than the study. However, in one published study that is accepted for milk sample analysis had reported recovers below 80%.[12] The authors stated that these recoveries

were acceptable because it was a multiresidue method. They further stated that because the values were in the range of -50% – 20% for the minimum trueness of quantitative methods according to the EU commission decision 2002/657 that they were acceptable.[12, 240]

The aim of this study was to assess the risk of milk in an estrogen mimic context; therefore, recoveries were corrected for when calculating the concentration of compounds in extracted milk samples. Some of the recoveries of the study were below 75% which can introduce skepticism because large errors are likely when correcting for recoveries. Whereas, when the recovery is close to 100%, there is more confidence in the corrected concentration because of the increased accuracy compared with low revoceries.[241] However, the recoveries obtained in this study are within the analytical acceptable range by not exceeding ± 20% for each recovery standard inject, which is a requirement stated in the Health and Food Safety (SANTE) report.[242] If more accurate recoveries are required, isotopically labeled compounds should be used. However, these compounds are expensive and hard to work with.[241] For completeness of a method this is something should be carried out because it adds to the robustness and reliability of the extraction method.

A possible reason for compounds having different recoveries is that the magnitude of matrix effects exhibited is different. It is possible that target compounds co-extract with other components of milk which limits the detection of target compounds through matrix suppression, therefore lowering the recoveries of target compounds.[17, 214] Although rare, target compounds can be enhanced with compounds which co-extract with target compounds.[213] Therefore it is very important that the fat globules are broken down; if not, compounds will not be released from the fat globules leading to low recoveries.[214] The selected method minimises matrix effects from proteins and fats through the de-proteination and clean-up steps carried out.

LOD and LOQ

It is frequently reported that xenoestrogens are biologically active at low concentrations; therefore, it is important that the LOD and LOQ concentrations are as

low as the analytical method allows.[84, 96, 105, 114, 188, 243] Therefore, analytical techniques which provide high sensitivity are important (e.g. HPLC-DAD, LC-MS). The analytical method used in the study was HPLC-DAD rather than LC-MS because data analysis of HPLC-DAD is quicker (discussed in Section 4.1). The important measure for determining the presence of a compound in a sample is the LOD. This is the lowest analyte concentration reliably distinguishable from a blank and at which detection is practical.[244] Whereas, the LOQ is the lowest concentration at which the analyte not only can be reliably detected but at which a predefined goal for bias and imprecision is met (e.g. defined UV spectrum).[244] The LOD and LOQ for the compounds of interest of the study were determined by diluting authentic standard in HPLC grade MeOH. The LODs and LOQs are shown in Table 3.2.

The compound which had the highest LOD was E2 with a concentration of 0.1 mg/L; the compound with the lowest LOD was daidzein at a concentration of 0.0025 mg/L. E2 also had the highest LOQ at a concentration of 1 mg/L; however, the compounds with the lowest LOQs were the phytoestrogens (i.e. formononetin, daidzein, equol) at a concentration of 0.05 mg/L.

The LODs of phytoestrogen recorded in this study are similar to previously published data using methods accepted for milk analysis. [158, 213, 220] These methods used HPLC, as well as the more sensitive LC-MS.[158, 213, 220] However, the study's LODs for zearalenone, EE2, BPA and E2 are not close to previously published LODs.[12, 14, 17, 21, 22, 212, 214, 216, 217, 245] The published studies having LODs 1,000 to 10,000 lower high than reported in this study.[12, 14, 17, 21, 22, 212, 214, 216, 217, 245] This is because LC-MS has much higher sensitivity compared with HPLC-DAD.

Milk is known to always contain endogenous estrogens, with the maximum basal concentration of these reported to be 3.3×10^{-5} mg/L.[208, 212] Cow's produce milk for their offspring which endogenous estrogens are important for the calves development; therefore, they will be present in the mother's milk.[208, 212] Therefore the analytical instrument used in the study was not sensitive enough to detect these low levels. The most suitable analytical instrument to detect the basal endogenous estrogen concentrations is LC-MS. The analytical method of this study could easily be adapted to be used with LC-MS which would increase the sensitivity of detection by orders of magnitude.

The director general responsible for the National Chemical Contaminants' Programme (NCCP) will often publish reports on milk and dairy products. The information in these reports will be provided by ESR who monitor milk and dairy products. "The NCCP was introduced to confirm that the control of chemical residues in milk and dairy products is appropriate and effective. It ensures the safety, wholesomeness, and the truth of labelling of milk and dairy products for both domestic and export markets."[246] Table 4.2 shows the residues which are monitored by this program.[246] In these reports an action limit is quoted, which is "the lowest allowable limit applied by New Zealand, Codex, and destination markets."[246] Another measure that is often quoted in these reports is the Limit of Reporting, which is "the lowest level at which residues will be reported as absolute numbers. It is equal to and higher than the LOQ."[246] Very rarely do these reports quote a LOD for method used; therefore, it is possible that compounds might be present but at a concentration below the action limit or Limit of Reporting. This is concerning because compounds might be present, but the population would not be told because it is below these limits. This could give a false sense of security to the population as many would assume that if it is below the Limit of Reporting, that the compound is not there at all, which might not be the case.

Phytoestrogens and some endogenous estrogens are not included on the list of residues that are monitored under the NCCP. The reason for this is because they are naturally present in pasture and the cow; therefore, the concentrations in milk are extremely hard to control. It is unreasonable to expect farmers to be able to control the amount of clover in pasture, and it would be almost impossible for the farmer to control the concentrations of endogenous estrogens in cow's milk. The NCCP understands this and therefore do not monitor them.

Table 4.2: The residues that are monitored under the NCCP

Registered agricultural compounds and veterinary medicines	Unregistered or prohibited agricultural compounds and veterinary medicines
Radionuclides	Organochlorines
Organophosphates	Dioxin and dioxin-like PCBs
Mycotoxins	Potentially toxic elements
Migration chemicals from food contact materials including packaging	Maintenance compounds
Possible adulterants	Any other chemical of national or international interest.
Any chemical compound or its metabolite that, via risk assessment or profiling, is considered to have the potential to adversely affect dairy material or product	

Although these compounds are hard to control, they should still be monitored; the endogenous estrogens in particular. The reason for this is because these compounds might have an effect on a consumer's circulating estrogen levels; for example, if milk has a high concentration of E1, this can be converted to E2. Thus, increasing the concentration of estrogens in circulation. The circulating concentration of estrogens are tightly regulated in the body because of their tight activity ranges; therefore, if endogenous estrogens are consumed and absorbed, the circulating estrogen concentration might increase. An increase in the circulating estrogen concentration could lead to an increase in the risk of breast cancer or decrease in sperm count. The reason for this could be because of the increased circulating estrogen concentration leads to an increased binding to ERs, and therefore, the responses elicited.

The mechanism of phytoestrogen estrogenicity is not well understood, which might be a reason why the NCCP does not monitor their concentrations. It is also possible that the ADIs for phytoestrogens might be high; therefore, the NCCP will not monitor them. It is possible that the ADI for a phytoestrogen might be so high that the cow would die in order to obtain the concentration in milk. As mentioned in Section 1.6.2, the ADIs for phytoestrogens are likely set high because the endpoint of the NOAEL toxicity

experiment might be a change in an enzyme concentration rather than a hormonal change. This would cause a big difference in NOAEL concentration because smaller concentrations are required to cause a hormonal change.

4.2 Assessment of the Concentration of Xenoestrogens in New Zealand Cow's Milk

To the best of our knowledge, this study is the first time that genistein, daidzein and BPA have been identified in New Zealand cow's milk sample extracts. These compounds were identified using a quick, easy, cheap and rugged method. In milk samples (n = 30) BPA was detected in 30% (9/30), daidzein was detected in 3.3% (1/30), and genistein was detected in 3.3% (1/30). The identification of these compounds was confirmed by co-chromatography with authentic standards and copying UV spectra using DAD. As previously discussed (Section 4.1.2) concentrations were corrected for recoveries to give a better estimate of actual milk concentrations to assess the risk of milk an estrogen mimic context. In this study, genistein was identified at a concentration of 0.062 mg/L after the treatment of sulfatase/β-glucuronidase enzyme from *H. pomatia.* Therefore, genistein was present in milk in a conjugated form; incubation with this enzyme broke this bond, releasing the aglycone. This is interesting because if genistein is present in its conjugated form in milk, when humans drink this milk, the gut microbiota could release the aglycone. This could then be absorbed into the blood stream where is could bind to ERs. Whereas, if genistein was present as the aglycone in milk, it would likely be conjugated by enzymes in the human gut and directed for excretion. Therefore, xenoestrogens present in their conjugated form in milk could be more concerning than when they are unconjugated.

This study is unlikely to be a true reflection of New Zealand cow's milk as a whole. It is more likely to show a local picture (i.e. Christchurch/South Island). The reason for this is because of milk pooling. Most dairy farms belong to a dairy co-operative (e.g. Fronterra) to give farms more power to demand better prices in the market. All farms part of the co-operative will send their milk to center owned by the co-operative. Here all of the individual farm's milk will be pooled together and mixed. The purpose of this

is to dilute any compounds with high concentrations and to make all the milk equal. This is not just to dilute unwanted compounds, but to make sure the nutritional value is also the same. These pooling centers are all over the country; for example, one in Christchurch, one in Auckland etc. Therefore, milk purchased in Auckland might be different to milk purchased in Christchurch. The pooling of milk is very good because it should balance and dilute any high concentrations of compounds, decreasing the risk to the consumer.

Milk sampling in this study was carried out by purchasing milk from local supermarkets in the Christchurch city region on different days. Expiry dates of milk were confirmed to be different to ensure they were from different batches. This study's sample size was small (n = 30) because this was a pilot study to evaluate if further monitoring is required. As mentioned previously, this study is likely to provide a snapshot of cow's milk from the South Island. To assess New Zealand cow's milk in its entirety, a larger sample size is required; for example, the number of samples should be above 100, with samples purchased from different cities in New Zealand throughout the whole year. This would provide the most complete picture of the state of New Zealand milk containing estrogen mimics. However, this study's findings provide a preliminary insight into the presence of endogenous estrogens and estrogen mimics in cow's milk. This could be extrapolated to milk across the country being as climate, regulations, and farming practices are likely to be the same. Any differences there might be is unlikely to cause a large variation to the findings in this study. The only event which could cause a difference would be if there was a natural disaster (e.g. earthquake, tsunami, flash flooding) which would lead to a cow being treated differently to affect its milk.

4.2.1 Xenoestrogens in New Zealand Cow's Milk

Phytoestrogens

Phytoestrogens were found in 2 out of 30 (7 %) cow's milk sample extracts from Christchurch. Genistein was found in one milk sample extract after the treatment of sulfatase/β-glucuronidase enzyme from *H. pomatia* at a concentration of 0.062 mg/L. Daidzein was identified in one milk sample extract at a concentration of 0.026 mg/L.

The presence of phytoestrogens was not consistent, and the concentrations were low. This was surprising because the major food source of New Zealand dairy cows is pasture, which contains red and white clover. Both of these species of clover are known to contain phytoestrogens.[155, 156] The most persistent clover species in New Zealand pasture is white clover because it is the most suited to the climate and growing conditions which are provided.[247] The major phytoestrogens in white closer are formononetin, biochanin A, genistein, and daidzein.[155] Therefore, New Zealand cows will consume these phytoestrogens from pasture; this could be transferred into the cow's milk. Other exposure routes for cows to phytoestrogens include soy-based feeds which are offered by famers throughout the year as an alternative to pasture. These feeds could be the major food source in the middle of seasons; for example, in winter because pasture is frosty and waterlogged, and summer when conditions are very dry, and pasture is dead. However, this is not the only alternative to pasture; farmers will choose the supplementation based upon the cheapest form of energy. Farmers might choose soy-based pellets because it can make lactating cows peak earlier with greater milk yields when it is included in the diet. These pellets will include phytoestrogens because they are made from soy, most commonly genistein, daidzein and glycitein; however, these are likely to be conjugated to glucose producing the respective glucoside compounds genistin, daidzin, and glycitin.[248] Once these glycosides reach the gut of cows, the glycosidic bond is broken by the microbiota, releasing the 'free' compounds where they can then be absorbed or metabolised.[248]

The compounds get into milk by crossing the blood-milk barrier which is likely analogous to the blood brain barrier; a highly selective semipermeable membrane barrier that separates the circulating blood. Interestingly, phytoestrogens have been shown to induce cell death of these membrane components.[249] This could cause the milk to mix with blood components, which could increase the risk of these components (e.g. phytoestrogens) getting into milk.[249] If this is the case, and phytoestrogens cross the barrier, it would be expected that phytoestrogens would be present in milk. In New Zealand, this concentration might be high because of the concentration of clover species in pasture.

Metabolism of Phytoestrogens

As alluded to previously, phytoestrogens are extensively metabolised by the microbiota of cows.[20, 155] These compounds are chemically altered by the addition of hydroxyl groups which, In turn, changes the compounds ability to be absorbed. The metabolism of phytoestrogens in cows was investigated by setting up a model rumen system. Cow rumen slices were collected from Silver Fern farms and incubated in Wilkins Chalgren broth spiked with formononetin (10 mg/L). The broth was extracted daily for 3 days. In this system, formononetin was suspected to be metablised to equol. This peak had the same t_R and UV spectrum as an equol authentic standard. The identity of the suspected equol peak was confirmed by spiking extracts with authentic standard. This caused an increased response of the peak when analysed by HPLC-DAD. This confirmed that the identity of the peak was equol. This finding indicated that the system is able to produce metabolites of phytoestrogens.

The results of the incubation of formononetin with cow rumen slices in the context of the risk milk has in an estrogen mimic context is interesting. As previously mentioned, equol is more estrogenic than its precursors (i.e. daidzein, formononetin, o-methyl equol).[158] Therefore, the study showing that equol is produced in the rumen is interesting because it means that compounds in pasture (e.g. formononetin) can be made more estrogenic; if equol can be transferred to milk, it would likely increase the estrogenic load. However, this study did not identify equol in New Zealand cow's milk sample extracts, which was surprising. It is possible that equol is not transferred across the blood-milk barrier; however, other published studies have identified equol in cow's milk extracts. It was also surprising that daidzein, another common phytoestrogen metabolite was not produced in the cow gut model. This was a surprise because daidzein is an intermediate to the production of equol; however, it is possible that the major route of metabolism to equol is via o-methyl equol instead of daidzein.[155, 157-160, 250] It is possible that the bacteria which carry out this conversion are not present in the samples. In the cow gut, bacteria are distributed differently to aid in digestion; for example, the bacteria on the rumen wall are likely to be different to the bacteria in the rumen liquid.[251] Different species of bacteria are likely to metabolise compounds differently or carry out different parts of metabolism. The different distribution of

bacteria makes assessing the system difficult as it is likely the only a snapshot of the bacteria is present in the complete system.

To the best of our knowledge, this is the first time that rumen slices have been used to show the cow's metabolism. Typically, rumen liquid is removed from cannulated dairy cows to show metabolism.[159, 250, 252] One possible difference between these systems are the bacteria present. The different distribution of bacteria makes assessing these systems difficult as it is likely the only a snapshot of bacteria is present in both system. The rumen is a complex system and investigation into cow gut metabolism needs to be continues. A more realistic system of the cow gut would be to combine the rumen liquid and rumen slices together.

The cow rumen samples used in the study were supplied by Silver Fern Farms, a livestock processing and marketing company. For this reason, livestock are completely cleaned by flushing the carcass with water. It is likely that bacteria which are loosely attached to the rumen wall are dislodged and washed out in this cleaning process. Therefore, it is possible that this system is not a complete representation of a cow gut. These samples were also exposed to an aerobic environment, which could cause some bacteria to die. The reason for this is that the gut is likely to be slightly anaerobic; therefore, the regular metabolism by the microbiome might not be carried out as not all of the microbiome is present in this system. It would be interesting to obtain cow rumen samples before the washing procedure to see if there are any differences in metabolite production between washed samples and unwashed samples. However, for an initial study into the metabolism of phytoestrogens in the cow gut, this is a good representation. Typically, researchers will use cell lines/models instead of live tissue samples to investigate metabolism; for example, the Caco-2 cell line from human small intestine which has been used to investigate drug metabolism.[253] Both systems have their benefits; live tissue is likely the best as it is closer to the real situation; however, it can be hard to access (e.g. human tissue). Whereas, cell lines may not be as close to the real situation as live tissue, but they are a good model for prediction and a lot easier to access (e.g. Caco-2 cells).

Modification of compounds is not the only route of metabolism. Another route of metabolism is through the addition of sulfate groups and glucuronides. The addition of these groups increases the compounds water solubility so that they can be absorbed in the blood stream and directed for excretion. Genistein was identified in New Zealand cow's milk after deconjugation by the sulfatase/β-glucuronidase enzyme from *H. pomatia*, which is consistent with published literature.[20, 157, 220] Thus, when genistein is consumed from the pasture, it is metabolised by conjugation in the cow's gut. Genistein can also be formed in the rumen of cows by the microbiota metabolising biochanin A from clover to genistein. Genistein is then bound to a glucuronide group or sulfate group, making the compound more soluble for excretion, or as demonstrated in this study, directed to the milk by crossing the blood-milk barrier.[12]

When humans consume milk containing phytoestrogen conjugates, the compound-conjugate bond is likely to be broken. The enzyme in the human gut capable of this is sulfatase and β-glucuronidase depending on the conjugate; thus, releasing the un-conjugated compound.[254] These compounds can be further metabolised by the gut microbiota or absorbed in to the blood stream. Interestingly, more than double of the non-conjugated compound is absorbed compared with the conjugated forms.[254] Therefore, it is possible that a dose of conjugates leads to a higher estrogenic load compared with a dose of non-conjugated compounds.

BPA

The findings of this study show the presence of BPA in New Zealand cow's milk samples. The concentrations measured in this study are low (0.012 – 0.066 mg/L) and of little relevance in an endocrine disruption context; however, it will likely be part of a cocktail. It is possible that BPA and other xenoestrogens will be additive because they have structural analogies with one another. BPA is capable of binding to ERs, as are other xenoestrogens; therefore, it is likely that they will have a sum of effects of the individual compounds. For this reason, it is important that residue concentrations are kept as low as possible. The source of BPA in cow's milk is likely to be coming from one of two sources. These two sources are either the production process of milk (e.g. milking equipment) or, from the packaging milk is sold in (e.g. milk bottles, milk

cartons). This study did not consistently identify BPA in milk sample extracts being identified in 9 of 30 (30%) milk sample extracts. If the source of this contamination was coming from either of these sources, a consistent appearance of BPA would be expected; however, as previously mentioned, New Zealand milk is pooled and mixed. Thus, any high concentrations of compounds are diluted during this process. Leaching of BPA from plastic equipment in farming equipment might be expected be consistent because equipment is only replaced when broken. Therefore, once the equipment started to leach BPA, it is likely to continue. However, BPA might only leach from new products for a short period of time. Whereas, leaching from milk packaging is likely to be more variable. For BPA to leach from plastic bottles it needs to be heated up. Milk is usually left in a fridge, otherwise it quickly goes off; therefore, leaching from bottles is unlikely.

Cows are milked using specialised equipment. This consists of a rubber lined teat cup which is attached to the cow's teat (Fig. 4.1). The teat cup creates a vacuum to mimic a calf suckling. A vacuum is important otherwise the cow will stop releasing milk. The milk travels from the teat cup through rubber tubing into a claw bowl. This is transparent and tough being made of industrial grade plastic (Fig 4.1). It is important that the claw is see-through so that farmers can see any issues with the milk (e.g. the presence of blood). It is also important that the claw bowl is tough because of the rough environment it is in. From the claw bowl, milk travels through rubber or silicone tubing into stainless-steel piping (Fig. 4.1). From the stainless-steel piping, milk enters a receiver can. Milk is then filtered and taken to stainless-steel bulk tanks (silos; Fig. 4.1). Milk is stored here until it was collected by milk tankers to take it to pooling centers.
Of the milking equipment mentioned above, the claw bowl is the only the piece of equipment possibly made of materials containing BPA. It also comes in direct contact with milk, giving opportunity to leach BPA. However, if the claw was the source, BPA might be present in all milk, even with milk pooling. This would likely be the case because all claw bowls should be made from similar plastics, despite manufacturer. However, it is most likely that this leaching will only be from new products. When plastics are synthesised with BPA monomers, 100% efficiency is unlikely; therefore, there will be 'free' BPA monomers which will leach into liquid for a period of time. Once all the 'free' BPA has gone, this product will stop leaching BPA, unless more is released (e.g. from heating up the plastic). This could explain the inconsistent presence

of BPA in cow's milk. The type of plastic used to make claw bowls was investigated by contacting Skellerup (a New Zealand dairy milk equipment supplier). A Skellerup employee was contacted 4 times regarding this. Unfortunately, this study's enquiry was passed onto the technical manager, who did not respond; and the general manage, who also did not respond. It is possible that Skellerup did not want to be interrogated about their products containing BPA or possible negative publicity associated with their products. Not receiving an email from the general manager or technical manager regarding the composition of their products indicated they do not want to be involved.

Figure 4.1: Specialised farming equipment found on a dairy farm. The rubber lined teat cup (**top left**) is attached to the cow's teat with a vacuum applied to mimic a suckling calf. The milk flows through the rubber tubing into a plastic claw bowl (**top left**). From here it flows though rubber or silicone tubing into stainless steel tubing and collected in a receiver tank (**top right**) before being filtered (**bottom left**) and stored in silos (**bottom right**) for collection by milk tankers.

The other possible source of the BPA in milk sample extracts is from the plastic bottles or cartons that milk is packaged in (Fig 4.2). Milk bottles and their lids are a soft semi opaque plastic which might use BPA monomers in the synthesis. Meadow Fresh (a New Zealand milk company) were contacted to ask the composition of their milk bottles. After a few emails, they stated that their bottles were 'made of food grade HDPE plastic' (Fig. 4.2). HDPE is a polyethylene thermoplastic made from petroleum, thus, BPA is not used in synthesis of the plastic milk bottles. Meadow Fresh were also questioned about the making of paper milk cartons. Milk cartons are a paper like product which is laminated so that the milk does not leak from the carton (Fig. 4.2). Meadow Fresh were a little more apprehensive to release this information; however, they confirmed that milk cartons are made of paperboard from wood chips, which has a waterproof layer applied, typically polyethylene. Therefore, based on the information Meadow Fresh provided about the composition of the plastic milk bottles and the paperboard cartons, BPA is highly unlikely to be coming from the packaging. The only other possibility is the seal which is underneath the cap (Fig. 4.2). This confirms that the milk has not been tampered with. There is the possibility that the plastic on the underside of the seal has a coating which uses BPA in the synthesis; this is very unlikely though because BPA is not used in any other packaging components.

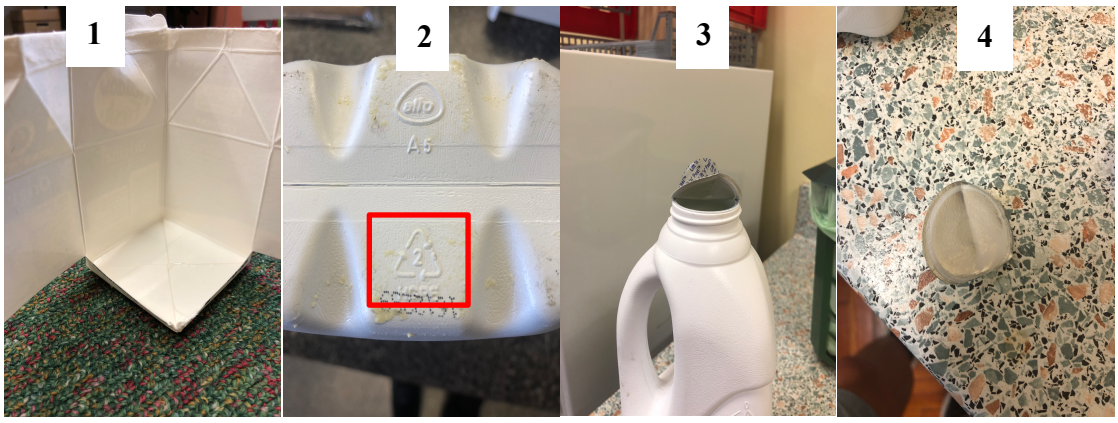


Figure 4.2: Packaging which cow's milk is sold in. Milk cartons (**1**) are made of paperboard with a PE coating on the inside, whereas milk bottles (**2**) are made of HDPE, which does not contain BPA, however, the seal on new milk bottles (**3**) may have a coating (**4**) of BPA which is leaching in to cow's milk.

It is possible that the BPA is not in the milk and instead is a contamination from lab equipment. It was well understood that BPA would be a contamination issue before starting milk sample extractions; therefore, extreme caution was taken to use as little plastic products as possible. In the early stages of milk sample extractions, single use sterile syringes were being used. When these extracts were analysed by HPLC-DAD a peak appeared in the same region as BPA. For this reason, it was decided to switch to glass syringes; when glass syringes were used instead, this peak disappeared. It was thought that the coating on the inside the of plastic sterile syringes was leaching into milk sample extracts, contaminating the sample. The only plastic product used which could not be avoided were 50 mL PP centrifuge tubes. No other members of the lab group had previously reported issues with BPA contamination using these centrifuge tubes; therefore, it was decided that they could be used and would not leach BPA. If BPA was a contamination of the lab, it would be present in every milk sample extract; however, it was only identified in 9 samples. Therefore, BPA was actually present in milk sample extracts.

Unknown Metabolite

Extracted milk samples analysed by HPLC-DAD often produced a peak with a t_R of 13.5 min. This peak had a UV spectrum similar to equol with slightly shifted adsorption maximum and eluted earlier than equol. It was suspected that this was a equol metabolite.

Unfortunately, no purchased authentic standards had the same retention time of this compound; therefore, the peak was investigated. Firstly, the peak was fraction collected using the HPLC-DAD set up. The residue was then analysed by direct inject probe mass spectrometry which identified an ion of 349.1263 m/z. The residue was also subjected to NMR to elucidate more information about the unknown peak. Unfortunately, the residue concentration was too low, and only solvent peaks were observed. The identity of the peak was hypothesized based on common metabolism pathways; for example, addition of hydroxyl groups, ring opening, addition of a sulfate or glucuronide.[250, 255] The addition of a glucuronide to equol lead to a mass which was too high for the mass spectrometry results, therefore it could not be a glucuronide metabolite. However, the addition of a sulfate group to equol lead to a mass of 322.33 (Fig. 4.3), which is close to the mass spectrometry result. Ring opening, with the addition of one and two

hydroxyl groups lead to masses of 340.35, 335.33, and 354.33 respectively (Fig. 4.3). Unfortunately, the correct mass could not be hypothesised for the residue. It is possible that this peak is not a metabolite of equol; instead it is an unrelated component of milk which has a similar UV spectrum to equol and a similar retention time as phytoestrogen metabolites.

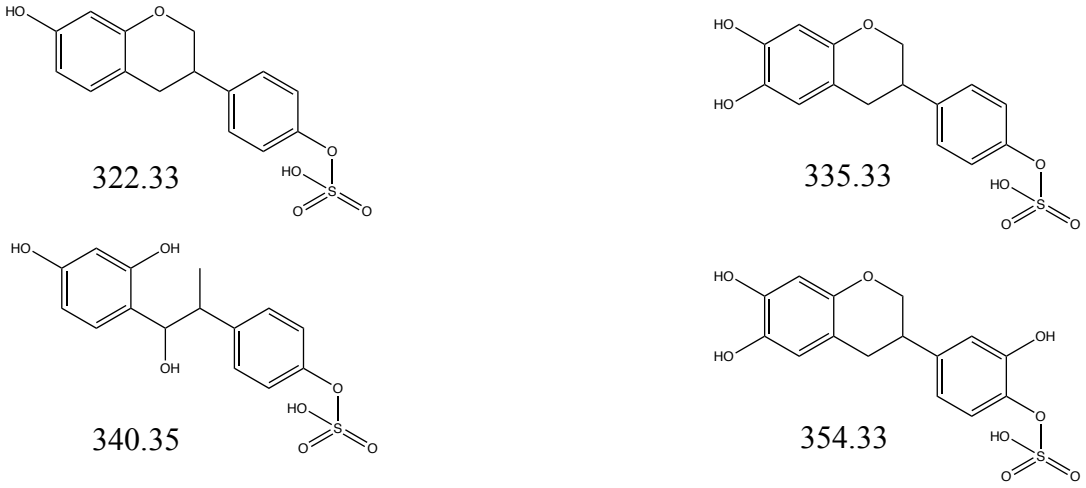


Figure 4.3: Hypothesised structures of potential equol metabolites. An unidentifiable peak (tR = 13.540) in HPLC-DAD was fraction collected and subjected to direct inject probe mass spectrometry. The unidentified metabolite has an ion mass of 349.126 m/z. None of the above hypothesised structures have the same m/z.

4.3 Concentration of Endogenous Estrogens and Xenoestrogens Across the World

An extensive literature search was carried out using Google Scholar, Web of Science, Scifinder, and Scopus using the key words; milk, estrogens, endocrine disruptors and liquid chromatography, dated from 1990 onwards to find all the data on measured concentrations of endogenous estrogens and xenoestrogens in cow's milk. From the references that were generated, these were further narrowed down based on 4 selection criteria: the concentration from milk samples; analytical results provided; recoveries above 70%; and internal standards used. 21 references met all selection criteria. Milk is produced and consumed in large quantities around the world; therefore, it was surprising and concerning that there appears to be little research around the possibility

of milk containing a high estrogenic load. Governments will closely monitor milk; however, it is unlikely that they monitor for phytoestrogens (discussed in Section 4.1.2) or publish these studies. As previously discussed, ADIs for phytoestrogens will likely be high because the toxicity data used for these are unlikely to be based on a hormonal change. Regulators will understand that estrogenic compounds work at concentrations lower than the action limits; however, they choose to ignore this because they see it as an acceptable risk. They are likely to be more concerned that the nutritional content is correct, as well as making sure that banned or highly regulated substances are not present in the milk. The reason for this is because milk cannot be exported if banned and highly regulated substances are present, or the nutritional value is not true to label. From the 21 references that met the selection criteria, concentrations of endogenous estrogens and xenoestrogens in cows varied depending on where in the world the milk was from (Table 3.1).

4.3.1 Phytoestrogens

The concentration of phytoestrogens (formononetin, equol, daidzein, genistein) appear to be higher in European countries compared with USA and China. A possible reason for this is that farming methods differ between Europe, and, USA and China. The differences may also be due to the difference in climate (e.g. rainfall). It is likely that all of these countries have different rainfalls throughout the year, with Europe likely to be the most consistent through the year, whereas, warmer temperatures would be expected in China and USA. This will affect when cows are able to graze pasture, and therefore, milk production. Farmers commonly supplement the cows diet with soy feed, silage, hay, and concentrate (carbohydrates, proteins, fats, minerals) to maintain the cow's nutritional requirements. Silage and soy feeds can be particularly high in phytoestrogens which could explain the high concentrations in Europe. The climates of USA and China in addition to the composition of pasture in these countries means that cows have a smaller exposure to xenoestrogens; this leads to lower concentrations of phytoestrogens in milk. However, the drier climates of USA and China may mean that in summers, farmers require supplementing feed in summer which would possibly increase the concentration of phytoestrogens in cow's milk.

The literature search identified that cow's milk produced in Australia contained very high concentrations of equol compared with other countries.[157] The source of the high concentrations of equol is likely to be from high exposures of formononetin and daidzein in their diet. The source of the high concentrations of these compounds are likely from red and white clover that is present in the pasture.[156-158] As previously mentioned (Section 1.6.1), bacteria in the cow's rumen can convert formononetin to equol via daidzein, which is likely reason for the presence of equol in Australian cow's milk.[158] Once equol has been produced, it might be conjugated via sulfation or glucuronidation and absorbed into blood. From here it can be transported and released into the cow's milk.[155, 157-160] It should be considered that it will not only be equol that will be present in its conjugated form; other phytoestrogens are likely to be absorbed from the cow's gut in their conjugated forms where they might be transferred to milk.[157] If phytoestrogens are present in cow's milk in their conjugated forms, humans would consume them. Once in the human gut, they might be deconjugated and absorbed.[254] If this occurs, this could be of concern because equol has a higher estrogenicity than its parent compounds (formononetin and daidzein).[158, 159] Therefore, equol could bind to ERs and illicit a response; for example, the upregulation of genes responsible for E2 synthesis. Whereas, if phytoestrogens are present in milk in their unconjugated form, then in the human gut they are likely to be metabolised by conjugation, where they are then directed for excretion. This removes the possibility that they could cause an estrogenic effect.

4.3.2 Endogenous estrogens

Endogenous estrogens in cow's milk follow a similar trend to phytoestrogens as discussed in section 4.3.1. Concentrations of endogenous estrogens are higher in Europe than the rest of the world, based on the findings of the literature review. The concentration of endogenous estrogens will depend on which trimester the cow is milked in.[12] It has been shown that when cows are milked in the final trimester, the total concentration of estrogens present in milk is three times higher than milk from the first trimester.[12] Unfortunately, this cannot be avoided; however, this could be taken into consideration by the farmer by not using milk from the final trimester when endogenous estrogen concentrations are at their highest. However, this is not a feasible option for a

farmer because the whole heard is likely to be in the same stage of lactation. This would cause the farmer to lose a large source of their income. One possible option is to split the heard into two, and offsetting when each heard would be in trimester 1 and 3. This would allow for mixing third trimester milk with first trimester milk to dilute the concentration of the endogenous estrogens in trimester 3 milk. In New Zealand, this is overcome by pooling milk, which dilutes milk from trimester 3.

4.3.3 Other estrogen mimics

Only 5 published literature met the requirements which were set that investigated other estrogen mimics (i.e. BPA, NP, zearalenone). The findings indicated that BPA can be present at high concentrations. In a study carried out in Italian cow's milk, BPA was found at a concentration of 0.5 mg/L. However, in China concentrations were measured at 4.90 x 10^{-4} mg/L. This difference could be down to the farming equipment used to milk cows or the milk bottles (Discussed in Section 4.2.1). It is possible that milk bottles in Italy at the time still contained BPA, which might lead to high concentrations in cow's milk. It is also concerning that there appears to be little research on the presence of mycotoxins in milk; only 1 research article was found to meet the set requirements of the literature review. The research article reported concentrations of zearalenone with a big range; it reported concentrations from 0.01 x 10^{-4} mg/L to 205 x 10^{-4} mg/L. Zearalenone is likely to enter milk by cows eating crops growing or stored in warm, humid climates contaminated with *Fusarium* fungi. The high concentration reported in milk might show the effect of cow's eating poorly stored, contaminated crops. The reason for the lack of data on mycotoxins in cow's milk might be because they are not commonly present in milk. The *Fusarium* fungi only grow in warm moist climates; therefore, these fungi might not thrive in many of the countries where mycotoxins have been investigated.

An interesting observation from the literature review was that as the fat content of milk increases (skim milk vs. whole milk), the concentration of xenoestrogens present was increased. This is likely because endogenous estrogens and xenoestrogens are fat soluble, therefore as the fat content of milk increased, more xenoestrogens can be present in the fat.[245] For example E2 in skim milk from Spain was found at a

concentration of 11 x 10^{-4} mg/L; whereas, in whole milk, E2 was found at a concentration of 54 x 10^{-4} mg/L. This suggests that drinking higher fat content cow's milk will mean a higher dose of endogenous estrogens and xenoestrogens is received; therefore, it may be a higher risk factor for breast cancer and decreased quality and quantity of sperm

4.3.4 This Study's Findings Compared with Data in the Scientific Literature

Phytoestrogens and xenoestrogens found in milk sample extracts in this study were similar to the concentrations of published data. This study identified the presence of genistein and daidzein in New Zealand cow's milk extracts. The concentration of genistein (0.062 mg/L) and daidzein (0.026 mg/L) was higher than the concentrations recorded in Australia (genistein = 0.022 mg/L), USA (genistein = 0.35 x 10^{-4} mg/L, daidzein = 0.24 x 10^{-4} mg/L), and Europe (genistein = 0.057 mg/L, daidzein = 0.016 mg/L).[19, 157, 213, 220] These concentrations are likely because of the high concentration of phytoestrogens that cows consume in their diet. Interestingly, this study did not identify equol, which was surprising. It would be expected that equol would be identified because of the high concentrations of phytoestrogens that New Zealand cows are expected to consume. New Zealand pasture contains high amounts of white clover that cows graze. This clover is known to contain the precursors of equol (daidzein, formononetin). Equol was identified in an Australian study at a high concentration (0.292 mg/L); this added to the surprise of not finding it in this study. It is likely that the species present in New Zealand pasture would be similar to Australia due to the similar conditions. The concentrations which genistein was found means it would be expected that equol would be present from the metabolism of genistein. It is possible that equol was present, but below the LOD for the analytical method. Formononetin was also not detected in this study. This is expected from literature, and the known metabolism routes. Formononetin is rapidly converted to equol by the cow rumen microbiota.[155, 157-160]

BPA found in New Zealand cow's milk extracts was found in 9 of 30 samples (30 %). The concentrations of BPA ranged from 0.012 mg/L to 0.066 mg/L. The concentrations measured in this study are similar to concentrations measured in Europe

(9.9 x 10^{-4} – 0.5 mg/L) and China (4.9 x 10^{-4} mg/L); however, the concentration range in this study was more precise compared with Europe.[21, 22, 214] A reason for this may be because in New Zealand the plastic milk bottles and cartons do not contain BPA and instead made of HDPE; whereas, in Europe at the time of the studies, BPA might of still been used as a monomer of the plastic. Therefore, it was possibly leaching into the cow's milk. However, as discussed in Section 4.2.1, the current materials used for milk packaging means that this is unlikely to be the source of BPA in cow's milk. Instead it appears more likely that equipment used during the milking and collection process are the source of contamination. The claw bowl is made of industrial grade plastic and possibly contains BPA. More investigation is required in to the milking equipment to locate where the BPA contaminating milk is from.

New Zealand's cow's milk appears to be safer in an estrogen mimic context compared with published data from other countries measuring the concentration of endogenous estrogens and xenoestrogens. This study did not find endogenous estrogens, mycotoxins or EE2. This is an important finding because these are more estrogenic than phytoestrogens. Therefore, suggesting that the risk of New Zealand cow's milk in an estrogen mimic context is lower compared with other countries. However, it is possible that these compounds are present, but are below the LODs for the method used in this study. This is almost certainly the case for endogenous estrogens which are reported to have a basal concentration of 3.3 x 10^{-5} mg/L.[208, 212] Another important finding of this study is that the phytoestrogens and xenoestrogens that were found in New Zealand cow's milk were not consistently present. This suggests that mixing milk from different farms is beneficial by diluting any unwanted compounds. Overall New Zealand milk appears to contain less endogenous estrogens and xenoestrogens compared with other countries based on this study.

4.4 Glyphosate

Glyphosate is the most used broad-spectrum herbicide in the world and is the active ingredient of Roundup. In New Zealand, farmers choose to seasonally spay Roundup on pasture to kill off remaining pasture and weeds so that new pasture can be planted. Whereas, in other countries, crops have been genetically modified to be resistant to

these herbicides. This allows farmers to spray their crops to kill off weeds without killing their crops. These crops are not allowed to be used in New Zealand because genetically modified crops cannot be grown here. The use of Roundup increases the efficiency of crops because they do not have to compete with weeds for important nutrients and space. Roundup require ~ 1 kg/hectare to be sprayed to kill unwanted wees; however, because New Zealand uses Roundup differently to the world, more glyphosate might be required to kill crops as well as weeds.[256] With controversy sounding the use and effects of glyphosate-based herbicides, it is important to understanding its risk to the environment, consumers and farmers.

All herbicides and pesticides have a withholding period which is the time between when a chemical is last used to when the food can be planted/harvested, or an animal can be slaughtered. This ensures the residue level does not exceed the ADI or other residues legislative parameters (e.g. MRL). Although this withholding period is in place it might be expected that residues are still present on food and in pasture. Recently, glyphosate residues were identified in ice cream from the United Kingdom at low concentrations (0.001 – 0.00123 mg/L), indicating that residues can be transferred from pastures to final prodct.[117] In New Zealand, glyphosate is used extensively used in farming; the cattle are given glyphosate sprayed pasture to graze; therefore, it is possible that cows are consuming pasture containing glyphosate residues, which might be transferred to the milk and tissues.

In 2015, the NCCP analysed milk samples for the presence of glyphosate residues. Here, it was reported that no glyphosate resides were found above the limit of reporting (0.05 mg/L).[257] This reporting limit is 10-fold more higher than concentrations reported in ice cream from the United Kingdom. In this study the presence of glyphosate in cow's milk was investigated using a high sensitivity method for the analysis. A method was used that had been previously accepted for the extraction and analysis of these residues in milk at concentrations as low as 0.001 mg/L.[209] The method used LC-MS; the benefit of this LC method was that the specific column used separated components based on their charge. These columns can also separate on hydrophobicity (i.e. normal phase and reverse phase). Unfortunately, LC-MS did not produce a mass ion consistent with glyphosate when an authentic standard was injected; however, when the same authentic standard was analysed by direct probe injection mass spectrometry, a mass

ion consistent with glyphosate was produced. Therefore, the unusual step to use direct probe inject mass spectrometry for the analysis of cow's milk was taken. This step was decided to be taken because when an authentic glyphosate standard at a concentration of 0.001mg/L was injected, a mass ion consistent with glyphosate was present. Therefore, it was decided that direct probe injection mass spectrometry was sensitive enough to detect the low levels which might be present in cow's milk. It was not known why the column could not separate and produce a mass ion consistent with glyphosate; one possibility is that it might bind to the column and not be released.

Five extracted cow's milk samples were analysed by direct probe inject mass spectrometry. In these 5 sample, none contained a mass ion consistent with glyphosate. However, by not using LC it is possible that matrix effects lead to suppression of the low concentrations of glyphosate in the cow's milk sample extracts, and therefore, no mass ion corresponding to glyphosate. LC would have been very beneficial as it would remove matrix effects by forcing glyphosate to loosely bind to the column and elute after the components which might suppress it. This is controlled by altering the composition of the mobile phase over time; which, in turn, causes compounds to elute due to their polarity. Using direct injection probe mass spectrometry means that an LOD or LOQ cannot be determined; therefore, one conclusion which can be drawn is that glyphosate is unlikely to be present in New Zealand cow's milk. The analysis of glyphosate in food products is currently 'trendy' because the complete toxicity profile is not known. A possible reason for the toxicity profile not being known is because the mechanism of action of glyphosate is also not known. This is a critical piece of information to understand the toxicity of glyphosate.

A possible mechanism of action of glyphosate is that it binds to ERs leading to a conformational change facilitating ER dimerization; which, in turn, could lead to an increase transcription of EREs. The ability of glyphosate to bind to ERα was investigated *in-silico* using Schrödinger Suite-Small-Molecule Drug Discovery Suite 2017-1. Using X-ray crystal structure coordinates for human ERα, rigid docking of glyphosate in the LBC of ERα was performed using Glide in XP mode, and a docking score was produced. Docking score approximates the ligand binding free energy, which includes contributions such as electrostatic and Van der Waals allowing for prediction

of binding energies. Actual binding energies are extremely hard to calculate *in-silico* and the discrepancies with actual values can be large; however, this is still a good tool for estimation. The more negative the score, the tighter the binding of a ligand. Glyphosate had a docking score of -21.55 kJ/mol compared with E2 which had a docking score of -41.76 kJ/mol. Therefore, glyphosate might be able to bind to the LBC of ERα, but not as well as E2. A reason for glyphosate not binding as strongly might be because it is shorter in length compared to E2; therefore, it would be unlikely to form the same bonds at each end of the LBC as E2. Figure 3.14 shows that glyphosate is capable of forming some of the same hydrogen as E2 does with the LBC of ERα; hydrogen bonds with Glu353, and Arg394 through a water molecule. However, glyphosate is not capable of forming a hydrogen bond with His524 like E2 does; this is located up to 8.67 Å and 9.90 Å away from atoms of glyphosate capable of forming these bonds. Hydrogen bonds have 3 categories, strong (2.2 - 2.5Å), moderate (2.5 - 3.2Å), and weak (3.2 - 4.4Å); thus, it is unlikely that hydrogen bonds would form between glyphosate and the key residues required for the binding of E2.[258] However, this study used rigid docking which means that the ERα is locked in the conformation which is optimal for E2 because this was the original ligand bound in the crystal structure coordinates. It is possible in a protein dynamics simulation that the binding of glyphosate may improve because of the flexibility and mobility of proteins in their natural environment. Glyphosate's binding to Glu353, and Arg394 through a water molecule might be enough to cause a conformational change which facilitates the dimerisation of the receptor leading to the translation and transcription of EREs. Protein dynamic modelling studies are the gold standard for *in-silico* investigations of the binding and interactions of ligands, however they require complex calculations carried by super computers. Rigid model docking is a good model for predicating whether a ligand has the potential to bind to ERα. Therefore, the risk of a compound in an estrogen mimic context can be predicted using Schrödinger software and the X-ray crystal structure coordinates of the human ERα.

4.5 Risk Assessment for New Zealand Cow's Milk

This study extracted and identified the presence of xenoestrogens in New Zealand cow's milk samples. However, the concentrations of the compounds found were low

and inconsistent; thus, they are probably irrelevant as risk factors to the associated effects of consuming estrogen mimics (e.g. increases risk of breast and prostate cancer, precautious puberty, decreased sperm count). It is likely that these compounds are part of a cocktail. These compounds have structural analogies; therefore, it is likely that they have additive effects on one another.

4.5.1 The New Zealand Population's Consumption of Cow's Milk

Cow's milk is an important part of the western diet from birth, offering important vitamins, nutrients, fats, and other essential components.[149] Cow's milk is a dietary staple in the western diet and consumption in Asian countries is increasing as western diets become more 'trendy'. For these reasons it would be expected that the daily consumption of liquid cow's milk would be relatively high because it is used for coffee, tea, cooking, breakfast, and drinking on its own. The most recent data on the New Zealand populations milk consumption was recorded in 2013 by Food and Agriculture Organization of the United Nations.[150] Here, it was reported that the amount of whole milk supplied per capita per day was 132.21 mL.[150] The reason for selecting whole milk is because that this figure will not include other milk related products (e.g. cheese, butter). This figure also assumes that every single person in New Zealand drinks milk, which is highly unlikely. This is unlikely because of people's allergies and diet choice meaning they do not drink cow's milk. Therefore, the real amount that a milk drinker in New Zealand will consume is likely to be slightly higher than 132.21 mL a day. However, this figure is for the amount of milk supplied per day per capita. New Zealand is the world's leading dairy exporter; therefore, New Zealand is likely to supply more than what is consumed. For this reason, the assumption that the New Zealand population consumes 100% of what is supplied is unrealistic; but, for the purpose of carrying out a risk assessment of this study's findings, the figure of 133 mL drunk per person per day will be used. This is because it is the most reliable and realistic amount that could be sourced; however, it is unlikely that large amounts of liquid milk are exported, dairy products are likely exported as solids.

Something which should be considered is that milk is used in other products and not only consumed in liquid form. Some examples of these are in chocolate, biscuits, infant

formula, cheese, butter, whey protein, sour cream, cottage cheese, dips, custard, and ice cream. Many people will consume at least one of these products daily, all of which use cow's milk or milk solids to be produced. The presence of xenoestrogens have been previously reported in a range of dairy products, with the products highest in fat (cheese and yoghurt) containing the highest concentration of xenoestrogens.[20, 207, 221, 228, 259-261] Some of these products use milk solids rather than liquid milk to be produced (e.g. chocolate), and depending on the company and where the product is made will determine where the milk solids are sourced. It is possible that the milk solids used for the production of these products are sourced from other countries because they are cheaper to buy, or it is where the product is produced. In this case it is unknown what the cows were fed, how the milk is stored or treated, the regulations of the country where the milk solids were made, and how the final products are produced. These unknowns can have a large effect on the concentration of endogenous estrogens and xenoestrogens present; for example, these milk solids could be from hot humid climates. In these climates, pasture which cows are fed could be contaminated with *Fusarium* fungi and poorly stored. This is likely to lead to zearalenone contamination which could then contaminate the milk by the cow consuming these residues. This could also be present in the milk solid, and therefore, the products which these are included in. Thus, the dose of estrogen mimics could be higher in products which include dairy (e.g. chocolate) compared with liquid cow's milk.

4.5.2 Estrogenic Dose of New Zealand Cow's Milk

This study identified genistein, daidzein, and BPA in cow's milk sample extracts. This is the first time that it has been published that these compounds have been identified in New Zealand cow's milk. Below, a risk assessment for these compounds at their calculated concentrations will be carried out for a person who drinks the New Zealand population per capita average amount milk (133 mL), and weight 62 kg.

Genistein

One cow's milk sample extract after the treatment of sulfatase/β-glucuronidase enzyme from *H. pomatia* was found to contain genistein at a concentration of 0.063 mg/L. This

would mean a dose of 8.246 x 10^{-3} mg would be received from drinking 133 mL of this milk. Genistein has a relative estrogenic potency (REP) of 0.03%, therefore the estrogenic dose received would be 2.47 x 10^{-4} mg if 100% of the dose was absorbed.[151] The ADI of genistein is 1.5 x 10^{-4} mg/kg bw, therefore for a 62 kg human, the dose is below the ADI, therefore if this person was to receive a dose of this concentration every day for the rest of their lives, no adverse effect would be expected to occur based on this ADI.[205] It is possible that genistein is metabolised in the human gut by the microbiota to more estrogenic compounds.

Daidzein

A single cow's milk sample extract was found to contain daidzein at a concentration of 0.026 mg/L. This would mean a dose of 3.458 x 10^{-3} mg would be received if 133 mL of this cow's milk was consumed. Daidzein has a REP of 1.0 x 10^{-4}%, therefore the estrogenic dose received would be 1.33 x 10^{-5} mg if 100% of the dose was absorbed.[151] There is no available ADI for daidzein; however, it is expected that it would be similar to equol (ADI = 20; endpoint = death) or genistein (ADI = 1.5 x 10^{-4} ; endpoint = hormonal effect); therefore, for a 62 kg human the ADI would not be expected to be exceeded.[205] Daidzein was only found in one sample. If the ADI set for daidzein was lower compared with equol and genistein, this could mean the ADI would be exceeded. However, it will still be expected that no effect would occur as this dose was only found in one milk sample. It should be considered that daidzein could be further metabolised by the microbiota in the human gut to more estrogenic compounds (e.g. equol)

BPA

BPA was found in 9 of 30 (30%) cow's milk sample extracts at concentrations ranging from 0.012 mg/L to 0.066 mg/L. Therefore, the doses received would range from 1.596 x 10^{-3} mg to 8.778 x 10^{-3} mg if someone drunk 133 mL of these cow's milk. The REP of BPA is 5.1 x 10^{-3} %, therefore the estrogenic doses ranges from 8.14 x 10^{-6} mg to 4.477 x 10^{-5} mg. The ADI of BPA 0.05 mg/kg bw, therefore the dose calculated is unlikely to cause any adverse effects for a 63 kg human.[206] It should be considered that in humans, BPA is rapidly absorbed from the gastrointestinal tract into blood.[262] It is then conjugated with glucuronic acid in the liver through first pass metabolism, which

is then rapidly cleared from the body by elimination in the urine.[262] Elimination usually occurs within 24 hours of oral doses.[262] Therefore, it is highly unlikely that any adverse effects with chronic or acute exposure would occur at the doses reported here.

Glyphosate

Glyphosate was not identified in 5 milk sample extracts analysed by direct injected probe mass spectrometry. The Ministry of Primary industries monitor milk for the presence of glyphosate, with published limit of reporting of 0.05 mg/L and limit of quantification of 0.01 mg/L.[257] If milk contained glyphosate at a concentration of 0.01 mg/L and 133 mL of milk was consumed, this person would receive a dose of 1.33 x 10^{-3} mg per day if the dose was completely absorbed. This dose is over 100-fold smaller than glyphosate's ADI of 0.5 mg/kg bw.[263] However, if glyphosate has the ability to bind to the ERs, or cause changes to signaling or protein expression related to hormones synthesis (e.g. change the concentration of aromatase), the concentration of 1.33 x 10^{-3} received daily for a lifetime could lead to adverse effects.

4.5.3 Worst Case Scenario of the Estrogenic Dose from New Zealand Cow's Milk

In this study genistein, daidzein, and BPA were identified in New Zealand cow's milk sample extracts; however, the other target compounds (E2, EE2, formononetin, equol, zearalenone) may have still been present, but below their detection limits for this study. Therefore, a risk assessment should be carried out based on the compounds being present at the LOD because this is the lowest concentration which a compound is present, although it cannot be quantified. Shown in Table 4.1 is a summary of the worst-case scenarios if the compounds of interest (E2, EE2, formononetin, genistein, daidzein, equol, BPA, zearalenone) for this study were found at their LOD. This was calculated using the New Zealand population's average milk consumption per capita of 133 mL drunk daily. If the data presented in Table 4.3 was the real situation for every bottle of milk, there should be a huge concern. The reason for this is because the three most estrogenic target compounds of this study (E2, EE2) exceed their ADI for a 62 kg human. Although the ADI is the concentration at which a compound can be ingested

every day for a lifetime with no effect; if this is exceeded for an extended period of time, it is possible that adverse effects may occur. If this was the real situation it would be expected that milk would be investigated to reduce the concentration of compounds. If this situation was so bad, milk might be withdrawn for sale until the concentrations were reduced. It is highly unlikely that this is the case, and that the LOD for the method used in this study are too high to detect the actual concentrations (discussed in Section 4.1.2).

There is a large assumption with most risk assessments that the whole population consumes a product, and that it is consumed equally; however, this is highly unlikely to be the case. A proportion of the New Zealand population wont drink milk for health reasons (lactose intolerance) or by choice (personal preference, vegan, upbringing, diet), a portion of the population will also drink more milk (personal preference, nutritional value e.g. calcium). Therefore, there is part of the population that will have no risk from cow's milk, and there will be part of the population which might have a higher risk. When a risk assessment is carried out this should be considered, especially for compounds which are close to their ADI at the populations average consumption figure. It should also be considered that not everyone is the same weight. People that weigh less than the 'average' 62 kg will likely be of a higher risk because their dose/kg bw will be higher.

Table 4.3: Summary of worst case scenario of New Zealand cow's milk if compounds were present at their LOD. The dose is calculated based on New Zealand population's milk consumption of 133 mL per day.

Compound	LOD (mg/L)	Dose (mg)	REP (%)	Estrogenic Dose (mg)	ADI (mg/kg bw/day)
E2	0.1	0.0133	100	0.0133	5×10^{-5} [203]
EE2	0.05	6.65×10^{-3}	91.15 [151]	6.061×10^{-3}	4.3×10^{-5} [204]
Formononetin	0.005	6.65×10^{-4}	0.000205 [264]	1.363×10^{-7}	N/A
Genistein	0.005	6.65×10^{-4}	0.03 [151]	1.995×10^{-5}	1.5×10^{-4} [205]
Daidzein	0.0025	3.325×10^{-4}	0.0001 [151]	3.325×10^{-8}	N/A
Equol	0.01	1.33×10^{-3}	0.07 [151]	9.31×10^{-5}	20
BPA	0.005	6.65×10^{-3}	0.0051 [151]	3.312×10^{-6}	0.05 [206]
Zearalenone	0.025	3.192×10^{-3}	0.63 [151]	2.011×10^{-3}	5×10^{-4} [203]

4.5.4 Is New Zealand Cow's Milk Safe?

Genistein, daidzein and BPA were identified in New Zealand cow's milk sample extracts. Genistein was found in one sample at a concentration of 0.063 mg/L, equating to an estrogenic dose of 2.47×10^{-4} mg. Daidzein was also found in one sample at a concentration of 0.026 mg/L, equating to an estrogenic dose of 1.33×10^{-5} mg. Whereas, BPA was found in 9 samples at concentrations ranging from 1.596×10^{-3} mg to 8.778×10^{-3} mg equating to estrogenic doses of from 8.14×10^{-6} mg to 4.477×10^{-5} mg. At the doses that these compounds were found, none exceeded their ADI for a 62 kg human. Based on the concentrations of the compounds found in this study, it is

expected that New Zealand cow's milk is safe and poses no risk to the consumer in an estrogen mimic context for someone who consumes the population average of cow's milk. As previously discussed, the whole population will not drink the same amount. Some of the population will drink more, and some will drink less based on preferences and health. This should be taken into consideration as some of the population will be receiving a higher does than expected, potentially putting them at a higher risk. It should also be considered that these compounds will be in a cocktail with one another. They have structural similarities and are likely to be estrogenic by a similar mechanism (i.e. binding to ERs); therefore, these compounds effects will likely be additive upon one another. This would therefore, increase the risk.

A worst-case scenario should be considered for the compounds that were not found above their LOD in this study. The LOD for E2, EE2, equol, and zearalenone are high compared with concentrations which would be expected for them to be present at in cow's milk. This is concerning because these (E2, EE2, equol, zearalenone) are the most estrogenic compound in their respective categories (endogenous estrogen, synthetic estrogen, phytoestrogen, mycotoxin respectively). If the compounds that were not found in this study were actually present at the method's LOD, the risk assessment would be very different. E2 and EE2 would exceed their ADI for a 62 kg human. The risk assessment from this study would change to advising that cow's milk should be highly monitored for E2 and EE2. If the concentrations of these compounds did not decrease below their ADI, milk may need to be withdrawn until the concentration of these compounds could be controlled.

4.6 Conclusions and Future Work

This study successfully used a method for extracting endogenous estrogens and xenoestrogens from cow's milk to determine the levels of these compounds in New Zealand cow's milk.

The extraction method was chosen based on three factors:

1. The compounds which were extracted
2. The ease and quickness of the extraction procedure
3. The reproducibility and quality of results

The recoveries of this method were varied being split into two groups; compounds which had low recoveries with BPA having the lowest (62%) and compounds which had high recoveries with EE2 having the highest (95%). The recoveries from this study are in line with previously published work that used other accepted methods for the extraction of endogenous estrogens and xenoestrogens from cow's milk.

The LOD and LOQ are important measures as they show the ability of the extraction and analytical methods. The LODs for the compounds of this study were varied with the lowest being daidzein (0.0025 mg/L) and the highest being E2 (0.1 mg/L). The LOQs followed a similar trend with formononetin, daidzein, equol, and zearalenone having the lowest (0.05 mg/L), and the highest being E2 (1 mg/L). The LODs for phytoestrogens were comparative with literature; however, for the endogenous estrogens, EE2, BPA and zearalenone the LODs were 1000 – 10,000-fold lower than reported in this study.

In the analysis of New Zealand cow's milk samples (n=30), BPA was found 9 times (30%), genistein once (3.3 %), and daidzein found once (3.3%). BPA was found at the lowest concentration of 0.012 mg/L and the highest concentration being 0.066 mg/L. The source of BPA is unlikely to be coming from the packaging because plastic milk bottles are made of food grade HDPE, and milk cartons are made of paperboard and a PE lining, none of which require BPA. However, some of the milking equipment, in particular the claw bowl, are made of plastic and the types of plastic used to make these

products could not be obtained due to a lack of response from a farming equipment supplier. Genistein was found at a concentration of 0.062 mg/L, and daidzein at a concentration of 0.026 mg/L. The likely source of these compounds is from the cow's diet; however, it was a surprise that equol (a known phytoestrogen metabolite) was not present in milk too. The presence of glyphosate in New Zealand milk samples was also instigated following it being found in ice cream in the United Kingdom. A previously accepted extraction method was used to determine the presence of glyphosate, due to the low concentrations published, direct inject probe mass spectroscopy was used. In New Zealand cow's milk samples (n = 5), no mass ion consistent with glyphosate was identified.

One aspect of this study that was not conclusive was the source of BPA; is a well-known endocrine disrupting chemical and the source of its presence in milk should be investigated. Efforts were made to contact companies to learn about their packaging and equipment, but no concise compounds or materials were provided by the equipment producer in particular. Therefore, future studies could further investigate the source of BPA by subjecting samples of plastics for infrared spectroscopy with appropriate sampling techniques.

New Zealand cow's milk appears safe for someone who consumes the New Zealand populations average amount of milk because BPA, genistein, and daidzein are below their ADI. It is possible that the ADI set was for an end point of an enzyme change (e.g. an upregulation of a liver enzyme) and not a hormonal change, which will be reflected in the ADI; therefore, it cannot be certain that there will no effect. However, these compounds are part of a cocktail. They are structurally similar having molecular similarities; therefore, it is possible that their effects are additive on one another. This could increase the risk of milk to the associated effects of consuming estrogen mimics. A worst-case scenario would be if the target compounds which were not found in this study (E2, EE2, formononetin, and zearalenone) were actually present at their LOD, this would set a new light on New Zealand cow's milk safety as E2, and EE2 would exceed their ADIs. A similar scenario may be the case if someone drinks more than the population's average consumption, or weighs less that the 'average', therefore regulators need to consider this when monitoring residues in milk.

This monitoring process of estrogenic compounds in milk should continue, and the method should be developed for LC-MS. Changing the analytical equipment should increase the sensitivity and therefore decrease the LOD and LOQ allowing for a more accurate measurement of estrogenic compounds.

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